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STUDIES ON THE GROWTH OF HUMAN MELANOMA CELLS IN VITRO

By

Y ROBERT BARISHAK[1]

Received 12 viii 60

In previously reported experiments (1) it was observed that tumour cells from human uveal melanomas could grow in tissue cultures for a limited period of time varying from a few weeks to 2–3 months. Attempts to establish a permanent line of cells in vitro in media of different compositions were all unsuccessful.

In the present publication observations on the effect of oxygen and carbon dioxide tension as well as of pH on the growth of cutaneous and ocular human malignant melanoma cells in vitro are reported. In addition some observations on the effect of para-phenylenediamine on human melanoma cells will be described.

Tumour tissue from nine patients with malignant melanomas of the skin (types mostly undetermined) and seven patients with malignant melanomas of the uvea was used. The uveal melanomas were of the following types: 1 spindle B (choroid), 1 epithelioid (choroid) and 5 mixed types (2 iris, 2 ciliary body, 1 choroid).

MATERIAL AND METHODS

The tumour tissue was grown in Carrel flasks and on flying coverslips in stationary tubes and in roller tubes. Clots prepared from cockerel plasma coag[illegible] with chick [illegible] composed o[illegible] and 70 per [illegible] centrations [illegible] a week. Mor[illegible]

For micr[illegible] fluid and s[illegible] [illegible]'s reticulum, hematoxylin eosin and Fontana Masson's stains were the routine methods.

For the study of fat and glycogen the Sudan III, Sudan IV, Sudan Black, the Best Carmine and P A S (Periodic acid Schiff's reagent) were used with a preference for the Sudan III IV mixture and P A S. Further technical details pertaining to the individual experiments will be given together with the results.

This work has been supported by grants from the Anders Hasselbalch Anti Leukæmia Fund, King Christian Xth Memorial Foundation and the Irma Foundation.

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RESULTS

As previously described (1) macrophages, fibroblasts and melanoma cells appeared in cultures of uveal melanomas In those of skin melanomas epithelial cells were also noted The melanoma cells were round (Fig 1 a), unipolar (Fig 1 b) bipolar (Fig 1 c) or multipolar (Fig 1 d) Intermediary forms were also frequently observed Mitoses were scarce (Fig 2) and could be seen only during the first 2–3 weeks following explantation No relationship could be determined between the histologic types of the melanomas and the number of mitoses encountered in the cultures Most of the melanoma cells coming out of the explants were unpigmented or slightly pigmented at the beginning However, in the course of one to two weeks increasing pigmentation was usually seen In some cultures this was already the case after one to two days of incubation

The Influence of Oxygen and Carbon Dioxide Tension on Melanoma Cells in vitro

Seven preliminary experiments were carried out with a modified gradient culture technique in order to study the influence of oxygen tension on the growth of melanoma cells Stationary tubes, containing 3 10 explants placed in a row on 50 mm long coverslips, were placed in a vertical position and filled with fluid medium, the distance from the upper and the lower explant to the surface of the medium being about 5 and 45 mm respectively After one week of incubation the coverslips were removed from the tubes, and the cultures were fixed and stained No gross difference between the growth of the upper and the lower explants could be demonstrated

In a second series of experiments cultures were gassed for 5 minutes, three times weekly with the following gas mixtures 5 per cent O_2 + 95 per cent N_2 (8 tubes) 15 per cent O_2 + 85 per cent N_2 (2 tubes), 50 per cent O_2 + 50 per cent N_2 (9 tubes), 5 per cent O_2 + 2 per cent CO_2 + 93 per cent N_2 (2 tubes), 15 per cent O_2 + 2 per cent CO_2 + 83 per cent N_2 (2 tubes), 20 per cent O_2 + 1 per cent CO_2 + 79 per cent N_2 (2 tubes), and 20 per cent O_2 + 5 per cent CO_2 + 75 per cent N_2 (2 tubes)

After ten days of incubation the cultures were fixed, stained and examined microscopically In the presence of 5 per cent CO_2 the fibroblasts seemed to be more proliferative, but otherwise no gross differences between the growth of the various cultures, were observed

In a third series of experiments the effect of continuous gassing with 50 per cent O_2 + 50 per cent N_2 and with 5 per cent O_2 + 95 per cent N_2 were studied The cultures were grown in open tubes placed in sterile gassing chambers Control cultures were grown at room air After three days of incubation the cultures were fixed and stained with the following techniques Hematoxylin-van Gieson, hematoxylin-eosin Fontana-Masson, Wilder's reticulum stain, P A S and Sudan II and IV

In these experiments 50 per cent O_2 seemed to have a stimulating effect on the mitotic activity The total number of mitotic figures in the individual growth zones was small, varying from 0–3 This does not necessarily mean that the mitotic coefficient (i e the number of mitoses per 1000 non-dividing cells) was small, but since only a limited number of cells appeared in the growth zones in most cultures, the mitotic coefficient could not be established However, in order to obtain a semi-quantitative estimate of the influence of oxygen tension on the mitotic activity, a comparative study of the number of explants, showing at least one mitotic figure relative to those showing no mitotic activity at all, was carried out

The results shown in table I indicate that 50 per cent O had a stimulatory effect on the mitotic activity as compared to room air (21 per cent O_2), while 5 per cent O_2 had no such effect

TABLE 1
The Influence of Oxygen Tension on the Mitotic Activity of 1–2 Days Old Cultures of Uveal Melanoma Cells

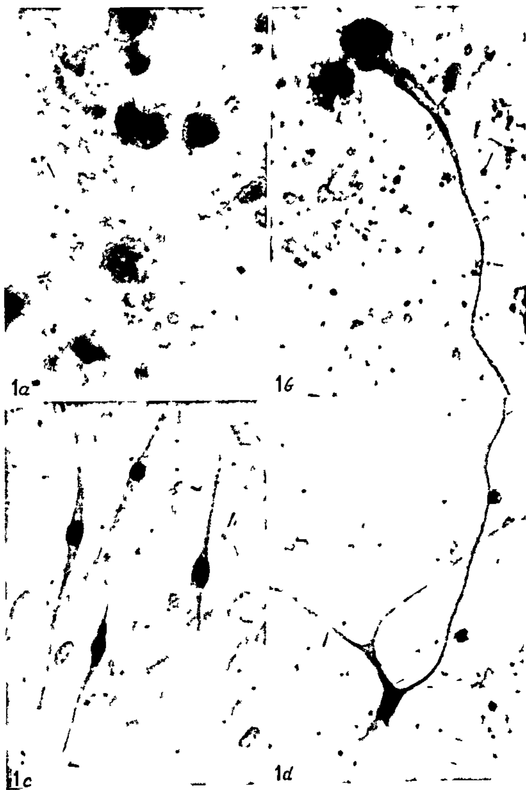
Gas phase	No of explants		Total
	+ mitoses	− mitoses	
Room air	2	31	33
50 % O_2 + 50 % N_2	27	35	62
Room air	3	51	54
5 % O_2 + 95 % N_2	3	47	50

This effect on the mitotic activity was mostly seen in young cultures and was less marked in cultures which were more than two days old when exposed to the various oxygen tensions (see Table 2) The mitotic figures were always observed close to the border of the explant, and usually in areas where no fibroblasts were seen

TABLE 2
The Influence of Oxygen Tension on the Mitotic Activity of 16 Days Old Cultures of Uveal Melanoma Cells

Gas phase	No of plants		Total
	+ mitoses	− mitoses	
Room air	2	43	45
50 % O_2 + 50 % N_2	8	49	57
Room air	1	47	48
5 % O_2 + 95 % N_2	2	49	51

1


1a
1б
1c
1d

As previously reported (1) melanoma cells are able to carry out active pigmentogenesis in vitro. Using Wilder's reticulum stain no gross differences in pigment formation could be detected between cultures grown at 5 per cent, 21 per cent and 50 per cent oxygen.

The storage of glycogen and fat was studied in cultures stained with P A S and with Sudan III and IV. No glycogen could be demonstrated, but the cells contained varying amounts of fat. However, the accumulation of fat seemed to be independent of the oxygen tension under which the cells had been grown.

The melanoma cells appeared as round cells, unipolar cells, bipolar cells, or multipolar or dendritic cells. These various forms may represent manifestations of a process of differentiation. The round cells were quite numerous in young cultures, while the multipolar cells were predominant in old cultures. 50 per cent O_2 seemed to stimulate the formation of multipolar cells (Fig 3 a, b) while 5 per cent O_2 had the opposite effect. At 5 per cent O_2 multipolar cells only appeared in the periphery of the growth zone (Fig 4), while similar cells were found in all parts of the growth zone at 50 per cent O_2.

The Influence of pH Variations on Melanoma Cells in vitro

For the study of the influence of pH on human melanoma cells in vitro 4–9 days old cultures were used. The pH values of the media were adjusted to 5.7 – 6.1 – 7.0 – 8.1 – 8.4 by the addition of primary or secondary phosphate. The media were changed once a week, and pH was controlled with the pH meter. After 15 days of cultivation, the cultures were fixed and stained.

A total of 54 explants from a skin melanoma and 141 explants from a uveal melanoma were used in this experiment. The main difference which was observed, was the predominance of bipolar and multipolar cells at pH 7 (Fig 5), while the round type of cells was more frequent at higher and lower pH values (Fig 5). Also intracellular fat accumulation seemed to be more conspicuous at pH 7 than at other pH values. Glycogen could not be demonstrated.

As mentioned before no mitotic activity could usually be demonstrated in cultures more than two weeks old. In the present experiments no cultures showed mitotic figures, indicating that the decline in mitotic activity is unrelated to changes in pH.

Figs 1 a–d

9 day old living coverslip culture of a malignant melanoma of the choroid (mixed type) (*Prot No 234/53*)

a Round tumor cells *Sudan III IV* stain × 450

b Unipolar melanoma cell Hematoxylin van Gieson stain × 300

c Bipolar melanoma cells P A S stain × 300

d Multipolar melanoma cell P A S stain × 240


1a
1б
1c
1d

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The storage of glycogen and fat was studied in cultures stained with P A S and with Sudan III and IV No glycogen could be demonstrated but the cells contained varying amounts of fat However the accumulation of fat seemed to be independent of the oxygen tension under which the cells had been grown

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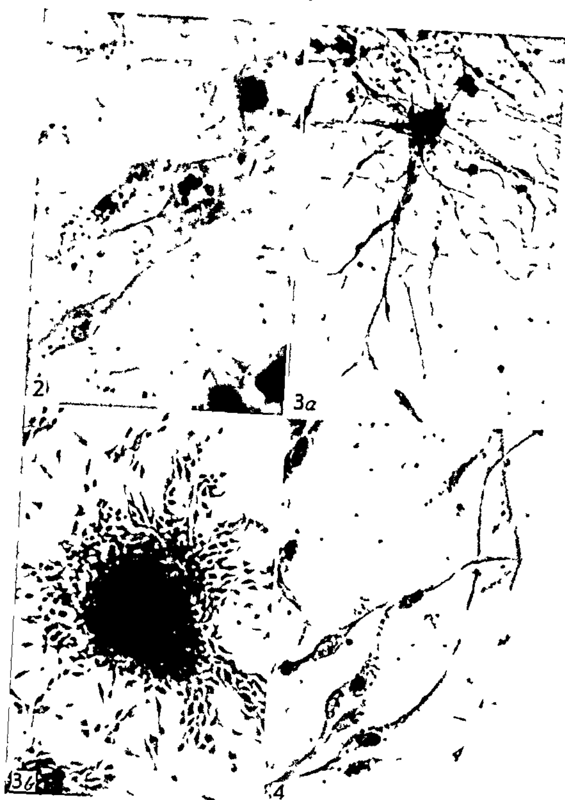
A total of 54 explants from a skin melanoma and 141 explants from a uveal melanoma were used in this experiment The main difference which was observed was the predominance of bipolar and multipolar cells at pH 7 (Fig 5) while the round type of cells was more frequent at higher and lower pH values (Fig 6) Also intracellular fat accumulation seemed to be more conspicuous at pH 7 than at other pH values Glycogen could not be demonstrated

As mentioned before no mitotic activity could usually be demonstrated in cultures more than two weeks old In the present experiments no cultures showed mitotic figures indicating that the decline in mitotic activity is unrelated to changes in pH

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9 days 11 R ... Fig 3 a 1

b 1 nig


2
3a
3б
4

Influence of Para Phenylenediamine on Human Melanoma Cells in vitro

Para phenylenediamine has been shown by *Riley* (9) to induce regression of S 91 mouse melanoma in vivo In the present experiment the cytotoxic effect of para-phenylenediamine at concentrations of 0 5 – 1 – 2 per cent was studied in 41 explants of skin melanomas and in 157 explants of uveal melanomas Freshly prepared para-phenylenediamine solution was added to the standard medium The cultures were 17 37 days old when exposed to the compound After one week of incubation they were fixed and stained The only effect of paraphenylenediamine which could be observed was an increase in cellular pigmentation (Fig 6 a, b) Signs of cell degeneration were not more conspicuous in treated cultures than in controls No intracellular crystal formation as described by *Kopac, Cameron & Chambers* (6) in normal and malignant rat liver cells, and by *Matoltsy* (7) in chick embryonal heart and skin fibroblasts, *in vitro*, was noted

DISCUSSION

A permanent strain of human uveal or cutaneous malignant melanoma cells could not be established by changing the O_2 and CO_2 tension or the pH of the media

However 50 per cent O_2 seemed to have a stimulating effect on the mitotic activity of melanoma cells, which contrasts with the results obtained by *Kieler* (3) on embryonic mouse heart myoblasts This author has found a marked reduction in the mitotic coefficient of these cells when they were exposed to a 50 per cent O_2 + 50 per cent N_2 mixture The different reactions of melanoma cells and myoblasts to high oxygen tensions might be due to differences in their metabolism This possibility, however, is not likely since, in this laboratory it has been shown that the metabolism of tumour cells is inhibited by high oxygen tensions

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Fig 2 Figs 3 a b and Fig 4

Fig 2 9 days old flying coverslip culture of a malignant melanoma of the choroid (same as Fig 1) The culture has been exposed for 3 days to continuous gassing with 50 per cent O_2 + 50 per cent N_2 Mitotic figure in a melanoma cell P A S stain × 450

Fig 3 9 days old flying coverslip culture of a malignant melanoma of the choroid (same as Fig 1)

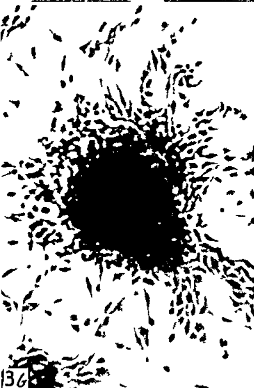
a The culture has been submitted to a continuous gassing with 50 per cent O_2 + 50 per cent N_2 for 3 days Numerous dendritic cells growing out from a small explant P A S stain × 100

b Control at room air Melanomatous growth note the closely packed short bipolar cells Sudan III IV stain × 100

Fig 4 10 day old flying cover[illegible]
(mixed type) (Pr[illegible]
gassing with 5 per cen[illegible]
phers of the plasma c[illegible]

2

3a





(2, 4, 5, 8) Also it should be mentioned that the fibroblasts which were frequently seen in the present experiments, seemed to be more proliferative under conditions, stimulating the growth of melanoma cells

As another possibility, it should be pointed out that the distance of diffusion through the fluid medium of the vertically arranged stationary tubes, may represent a limiting factor If this is the case the cells might be suffering from a relative lack of oxygen at 5 per cent O_2 and 21 per cent O_2 Thus, it is conceivable that in the present experiments the metabolism of the melanoma cells at 5 and 21 per cent O_2 has mainly been of the glycolytic type, while at 50 per cent O_2 the respiratory metabolism would also have played a role This would not only explain the effect of 50 per cent O_2 on the mitotic activity but also the differences in cell differentiation between cultures grown at various oxygen tensions

The experiments did not show any difference in the mitotic activity which could be related to the difference in melanoma types

SUMMARY.

The effect of oxygen and carbon dioxide tension as well as the effect of the pH upon the growth of human uveal and cutaneous melanomas was studied 50 per cent O_2 + 50 per cent N_2 was found to have a stimulating effect on the mitotic activity of melanoma cells in young cultures 1–16 days old

Melanoma cells seemed to show a higher rate of differentiation at pH 7 than at more acid or alkaline values

Studying the influence of para-phenylenediamine on melanoma cells in vitro, an increased pigmentation was the only effect observed

REFERENCES

1 *Barishak Y R* [illegible]

2 *Fisher A* Some [illegible] growth of [illegible]

Figs 5 a b and Figs 6 a b

Fig 5 19 day [illegible] the choroid (mixed t[illegible]

a The [illegible] shape of the melanoma [illegible] toplasm Sudan III [illegible]

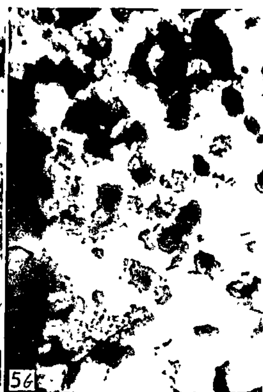
b The pH of the media has been adjusted to 5 7 Note the round shape of the melanoma cells Wilder's reticulum stain × 300

Fig 6 17 day old flying coverslip culture of a malignant melanoma of the choroid (same as Fig 1)

a Moderately pigmented bipolar melanoma cells Control Hematoxylin van Gieson stain × 450

b The culture has been exposed to 0 05 per cent P P D A solution for one week Heavily pigmented bipolar melanoma cells Hematoxylin van Gieson stain, × 450


5a


5б


6a


6б

FROM THE DEPARTMENT OF HISTOLOGY, UNIVERSITY OF UPPSALA, SWEDEN
(HEAD: PROF. MARTIN WRETE, M.D.)

AUTORADIOGRAPHIC ANALYSIS OF THE ACCUMULATION OF LYMPHOCYTES IN WOUNDS

By

KARL-ERIK FICHTELIUS and HANS DIDERHOLM

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In a previous article (*Fichtelius & Olerud* 1960) is was made probable that lymphocytes formed before the onset of the healing process were mobilized via the blood and accumulated in the wound. This conclusion was based indirectly on measurements of the specific activity of desoxyribonucleic acid (DNA) in the wound and control skin after administration of P^{32}. In this article a similar conclusion is reached with the aid of labelling with H^3-thymidine and subsequent autoradiography, which allows observations on the cellular level.

... the cell ...

MATERIAL AND METHODS

Four 2 months old male mice were used weighing 18–20 g. 0.5 μC H^3 thymidine per g body weight (spec. act. 2.7 C/millimole, Schwartz lab. New York) was injected intraperitoneally daily for 4 days. On the 7th day of the experiment 2 parallel incisions were made on the right side of the back. Each incision was about 1 cm long and closed with one suture. On the 12th day of the experiment, when there was a marked accumulation of lymph... and the wound a... 10 per cent forme... which had been ...

... was entirely filled with ... were ...

Cells were ... number ... sections ...

The ... supported by the *Swedish Medical Research Council*

3 *Kieler J* The cytotoxic effect of nitrous oxide at different oxygen tensions Acta pharmacol et toxicol *13* 301 308 1957
4 *Kieler J* Cultivation of Leukemia cells in the Cartesian Diver The Leukemias etiology pathophysiology and treatment Academic Press Inc Publishers New York N Y 1957 p 215 226
5 *Kieler J* Studies on the oxidative metabolism of the Yoshida ascites tumour cells VIIth International Cancer Congress London 1958 Acta UICC *16* 1079 1083 1960
6 *Kopac M J Cameron G & Chambers R* Neoplasm studies X The effects in tissue culture of some split products of p dimethylaminazobenzene on rat liver tumors Cancer Res *3* 290 292 1943
7 *Matoltsy A G* Studies on the action of P P D A on living cells J invest dermatol *21* 447 455 1953
8 *Nissen N I* The influence of oxygen tension on the respiration of the Ehrlich ascites tumour cells Soc Exp Biol 112th Conference København 1959
9 *Riley V* The melanoma as a model in a rational chemotherapy study Pigment Cell Biology Academic Press Inc Publishers New York 1959 p 289 433

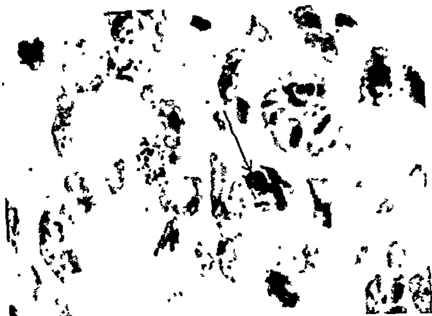


Fig 1
Labelled fibroblasts in the wound. A labelled lymphocyte at the arrow

TABLE 2
The Labelling of Fibroblasts and Epidermal Cells in Control Skin and in the Wound

Number of animal		Control skin		Wound	
		Fibroblasts	Epidermal cells	Fibroblasts	Epidermal cells
1	Labelled cells/view field	8.4	11.8	9.5	13.8
	Grain count/labelled cell	5.8	4.5	5.2	4.4
	Total grains counted in labelled cells	980	1063	988	1206
2	Labelled cells/view field	2.9	4.8	7.8	12.2
	[illegible]	[illegible]	[illegible]	[illegible]	5.5
	[illegible]	[illegible]	[illegible]	[illegible]	1342
3	[illegible]	[illegible]	[illegible]	[illegible]	8.9
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	Total grains counted in labelled cells	447	355	563	663

The Labelling of Fibroblasts

The labelled cells in corium other than "round cells" were mainly fibroblasts as defined by *Finerty & Cowdry* (1960) and comprised 75 per cent of the labelled cells in corium of control skin and 55 per cent of the labelled cells in corium of the wound. From Table 2 it is evident

RESULTS

The Labelling of "Round Cells"

Typical lymphocytes and similar cells with a small, dense, deeply stained nucleus, corresponding to macrophages as described by *Finerty & Cowdry* (1960) were classified as "round cells" In addition a smaller number of plasma cells, and probably also monocytes were included in this group From Table 1 it is evident that the number of labelled "round cells" per viewfield was higher in the wound than in control skin This was valid for both epidermis and corium in all 4 animals This increase of the number of labelled "round cells" per viewfield can be explained by the lively cellproliferation in the wound and the consequent distribution of the label to more cells However, the total number of grains in labelled "round cells" was higher in the wound than in control skin in all cases Since there is no free thymidine available for DNA-synthesis during the wound healing, (which started more than 3 days after the last injection of thymidine), the increase in total label means, that the *additional label of the "round cells" has been brought to the wound* from outside—probably by nucleated blood cells This additional label may originate directly from accumulating mononuclear blood cells, which were labelled before wounding Another explanation is that the DNA of labelled nucleated blood cells was reutilized at the formation of "round cells" within the wound Such a reutilization of DNA should give a lower grain-count in newly formed "round cells" than in the cells from which the labelled DNA was reutilized The level of the label was, however, too low in this experiment to allow an estimation of the decrease in grain count (see Table 1) This renders it more difficult to tell whether there was a simple accumulation of lymphocytes from the blood or a reutilization of DNA of nucleated blood cells

TABLE 1

The Labelling of "Round Cells" in Control Skin and in the Wound

Number of animal		Control skin		Wound	
		Corium	Epidermis	Corium	Epidermis
1	Labelled cells/viewfield	1.7	0.8	5.9	3.3
	Grain count/labelled cell	6.5	3.7	4.8	3.7
	Total grains counted in labelled cells	221	55	560	241
2	Labelled cells/viewfield	1.2	1.8	7.9	5.5
	Grain count/labelled cell	7.7	6.6	5.5	4.8
	Total grains counted in labelled cells	177	231	873	533
3	Labelled cells/viewfield	1.2	1.1	6.6	5.3
	Grain count/labelled cell	5.2	4.5	4.2	4.2
	Total grains counted in labelled cells	119	98	559	443
4	Labelled cells/viewfield	0.6	0.7	5.5	2.7
	Grain count/labelled cell	4.6	3.8	4.0	3.8
	Total grains counted in labelled cells	51	50	437	206

before wounding and which transformed to epidermal cells Another explanation is that the DNA of labelled nucleated blood cells was reutilized at the formation of epidermal cells within the wound

Discussion of the Method

In this experiment one viewfield in the wound was compared to one in the control skin The distance between two structures within the intact skin increases when an incision is made in the immediate vicinity of these structures, partly due to edema and partly due to cell proliferation If shrinkage during the histological procedures is proportionally the same in the wound and in control skin, then a smaller part of the original tissue will be seen in a viewfield of the wound than in a viewfield of the same tissue had no incision been made The shrinkage may be porportionally greater in the wound than in control skin, but it is [illegible] could compensate for the in[illegible] similar experiments in guinea [illegible] s 250 per cent of that of an equally large piece of control skin (*Fichtelius & Olerud* 1960) The conclusion is thus that a viewfield in the wound comprises only a part of the original tissue This is an error which strengthens our main con[illegible] n outside [illegible] the wound, only [illegible] nerally speaking grain count per cell is halved at mitotosis, and the more mitoses in a tissue the less the grain count cell will become with time As a consequence the grain count cell is lower in the wound where there is an increased frequency of mitosis In the wound the label in more cells has been diluted to such an extent that the daughter cells are no longer registered as labelled The loss in the total number of grains registered is thus greater in the wound than in control skin Again this is an error of the method which strengthens our main conclusion that label has been brought to the wound from outside

Discussion of the Results

Irrespectively of whether our results indicate a transformation of cells or a reutilization of DNA, they have confirmed the results of the earlier experiments with P^{32} (*Fichtelius & Olerud* 1960) The aim of the present experiment was only to complement the preceeding experiment on the cellular level and hence the small series With regard to the congruence of the results of the preceeding and the present experiment and to the great difference in label of the "round cells" of the wound and control skin (the increase of grains over labelled "round cells" was 5 2 times in corium and 3 8 times in epidermis), the conclusion that label was brought to the "round cells" of the wound from outside must be considered very likely



Fig 2
Labelled epidermal cells in the wound

that the number of labelled fibroblasts per viewfield was higher in the wound than in control skin in all cases See also Fig 1 The total number of grains in labelled fibroblasts was also higher in the wound than in control skin in all cases (The difference was, however, very small in the first animal) That indicated that label had been added to the fibroblasts from outside, probably via nucleated blood cells This additional label may originate directly from accumulating lymphocytes, which were labelled before wounding and which transformed into fibroblasts Another explanation is that the DNA of labelled nucleated blood cells was reutilized at the formation of fibroblasts within the wound

The Labelling of Epidermal Cells

The labelled cells in epidermis other than "round cells were all epithelial cells and comprised 87 per cent of the labelled cells in epidermis of control skin and 75 per cent of the labelled cells in epidermis of the wound From Table 2 it is evident that the number of labelled epidermal cells per viewfield was higher in the wound than in control skin in all cases See also Fig 2 The total number of grains in labelled epidermal cells was also higher in the wound than in control skin in all cases That means that label has been added to the epidermal cells from outside, probably via nucleated blood cells This additional label may originate directly from accumulating lymphocytes, which were labelled

epidermal cells yielded by the present experiment may provide a better understanding of the mechanism of sensitization in delayed type of allergy and the mechanism of passive transfer of such sensitivity (*Chase* 1946 *Haxthausen* 1917 *Hagerman* 1954 *Fichtelius* 1959)

SUMMARY

A relatively small fraction of the lymphocyte population in mice was labelled with four daily injections of H³ thymidine Three days later when free thymidine was nondetectable a wound was made on the mice and after an additional five days when there was a marked accumulation of lymphocytes in the wound the animals were sacrified and the wound and a symmetricly located area of the animal skin was examined autoradiographically It was shown that there were more labelled "round cells in the wound than in control skin and that the additional label of the round cells was brought to the wound from outside—probably via nucleated blood cells In addition it was shown that the labelling of the fibroblasts and epidermal cells was higher in the wound than in control skin The significance of this finding is discussed with regard to a possible trephocyte function of the lymphocytes and their transformations into fibroblasts and epidermal cells

REFERENCES

Andreasen F On the Occurrence of the Lymphocytes in the Normal Epidermis Acta Dermato Venereologica 32 17 21 1952

Andreasen F Observations on Proliferation and Death of Lymphocytes in The Kinetic of Cellular Proliferation p 19 21 ed Stohlman F Jr New York and

19[illegible]

Donarch I & Pele S R Autoradiographic Technique Brit J Radiol 23 184 19? 1950

Fichtelius K F The Influence on Lymphocytes of Sensitization to Dinitrochlorobenzene Shown by Transfusion of Labelled Lymphocytes Acta Dermato Venereologica 3? 7 81 1959

Fichtelius K F & Olerud S To be published in Acta path et microbiol scand nav 1961

Fnerty J C & Cowdry F V Connective Tissues in A Textbook of Histology p 1?4 159 Philadelphia Lea & Febiger 1960

Hagerman C How is Epidermal Hypersensitivity Transmitted through Lymphocytes Acta Dermato Venereologica 34 51 56 1954

Haxthausen H Studies on the Role of the Lymphocytes as "Transmitter" of the Hypersensitiveness in Allergic Eczema Acta Dermato Venereologica 27 275–286 1917

Hughes W L Bond [illegible]
Sherman F C [illegible]
graphy with [illegible]
[illegible]

The conclusion, that label was brought also to fibroblasts and epidermal cells in the wound from outside, is, however, less certain The increase of grains over fibroblasts was only 1.7 times and over epidermal cells 1 6 times There was, however, an increase in all four animals in both fibroblasts and epidermal cells of the wound and the two errors of the method discussed above tend to diminish possible differences These data make it probable that label was brought from outside also to fibroblasts and epidermal cells of the wound

Our experiment does not indicate whether mononuclear blood cells transform into fibroblasts or epidermal cells in the wound, or whether DNA of these cells (perhaps including granulocytes) is reutilized on some level for the purpose of fibroblast or epidermal cell formation in the wound

A large body of conflicting literature exists on the transformation of lymphocytes and monocytes into other cells *Medawar* (1957) writes "beyond the central nervous system, there can be few cells into which lymphocytes have not been alleged to transform themselves" Some authors state that lymphocytes and monocytes can be transformed into fibroblasts The literature is reviewed by *Yoffey & Courtice* (1956) and *Trowell* (1958) However, real evidence that lymphocytes or monocytes can transform into fibroblasts is lacking

There is general agreement that lymphocytes are not lost on the surface of the epidermis after migration through it However, the ultimate fate of lymphocytes in the epidermis is not clear *Andrew & Andrew* (1949) state that there is no evidence to indicate that lymphocytes degenerate within the epidermis, but that the lymphocytes are transformed into ordinary epithelial cells On the other hand *Andreasen* (1952) claims to have seen lymphocytes degenerating in the epidermis and states that the epidermis is one of the disposal areas for the lymphocytes of the body

The extent and level at which DNA is reutilized for cell proliferation is far from clear The reader is referred to a discussion on this subject in *Stohlman*'s "The Kinetics of Cellular Proliferation" (1959) If our results can be explained by DNA reutilization as we are inclined to believe, it is an important contribution to this discussion, because, as far as we know, a reutilization of labelled DNA has not been shown to occur in such a way that the reutilized labelled DNA can still be visualized in autoradiographs

A reutilization of lymphocyte-DNA for cell proliferation in a wound fits very well to the old idea of *Alexis Carrel*, that lymphocytes supply something necessary for tissue growth This trephocyte function for lymphocytes has found many supporters during the past years and has been advocated recently by *Kelsall & Crabb* (1958) and *Andreasen* (1959), but it has not been shown to exist A reutilization of lymphocyte-DNA also fits very well into the hypothesis of *Medawar* (1957) that nucleic acids circulate in the body The information on the labelling of

dermal cells yielded by the present experiment may provide a better derstanding of the mechanism of sensitization in delayed type of ergy and the mechanism of passive transfer of such sensitivity (*Chase* 46, *Haxthausen* 1947, *Hagerman* 1954, *Fichtelius* 1959)

SUMMARY

A relatively small fraction of the lymphocyte population in mice was belled with four daily injections of H^3-thymidine Three days later, hen free thymidine was nondetectable, a wound was made on the ice, and after an additional five days, when there was a marked accu- ulation of lymphocytes in the wound, the animals were sacrificed and e wound and a symmetricly located area of the animal skin was amined autoradiographically It was shown that there were more belled "round cells" in the wound than in control skin, and that the lditional label of the "round cells" was brought to the wound from itside—probably via nucleated blood cells In addition, it was shown iat the labelling of the fibroblasts and epidermal cells was higher in ie wound than in control skin The significance of this finding is iscussed with regard to a possible trephocyte function of the lympho- ytes and their transformations into fibroblasts and epidermal cells

REFERENCES

ndre

ndr

Indr

hase

Doniach I & Pelc S R Autoradiographic Technique Brit J Radiol 23 184–192 1950

Fichtelius K F The Influence on Lymphocytes of Sensitization to Dinitrochloro benzene Shown by Transfusion of Labelled Lymphocytes Acta Dermato-Vene reol scand 39 75 81 1959

Fichtelius K F & Oleru I S To be published in Acta path et microbiol scandinav 1961

Finerty J C & Cowdry E V Connective Tissues, in A Textbook of Histology, p 124 159 Philadelphia Lee & Febiger 1960

Hagerman C ... cytes Acta D ... Lympho

Haxthausen H ... Hypersensitiv ... r" of the 256 1947 ... 27 275–

Hughes W L ...

Kelsall ...

McL ...

The *conclusion, that label was brought also to fibroblasts and epidermal cells in the wound from outside*, is, however, less certain. The increase of grains over fibroblasts was only 1.7 times and over epidermal cells 1.6 times. There was, however, an increase in all four animals in both fibroblasts and epidermal cells of the wound and the two errors of the method discussed above tend to diminish possible differences. These data make it probable that label was brought from outside also to fibroblasts and epidermal cells of the wound.

Our experiment does not indicate whether mononuclear blood cells transform into fibroblasts or epidermal cells in the wound, or whether DNA of these cells (perhaps including granulocytes) is reutilized on some level for the purpose of fibroblast or epidermal cell formation in the wound.

A large body of conflicting literature exists on the transformation of lymphocytes and monocytes into other cells. *Medawar* (1957) writes: "beyond the central nervous system, there can be few cells into which lymphocytes have not been alleged to transform themselves". Some authors state that lymphocytes and monocytes can be transformed into fibroblasts. The literature is reviewed by *Yoffey & Courtice* (1956) and *Trowell* (1958). However, real evidence that lymphocytes or monocytes can transform into fibroblasts is lacking.

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RESPIRATORY TRACT INFLAMMATION AFTER EXPOSURE TO FIRE SMOKE

By

GÖRAN SKÖLD and ULF BRUNK

Received 25 xi 60

The inflammatory changes sometimes seen in the respiratory tract at post-mortem examination of victims of explosions or fire accidents have long been regarded as the effect of the heat on the mucosa (*Harbitz, Foerster, Goldbach*). However, judging from the case described below and from subsequent animal experiments, it would appear that the respiratory tract changes need not always be due to such heat but may also be caused by chemical irritation (fire smoke).

REPORT OF CASE

Two women, one aged 72 and bed [illegible]
cottage. Early one morning the [illegible]

[illegible]

The hall was full of smoke. The [illegible]
door. They could see *flames in th*[illegible] They
carried the woman out into the *open air*, where she soon recovered and told her
neighbours that she had been awakened by the older [illegible]
to the smoke. Since she had always g[illegible]
she did so this time too. When she [illegible]
out the plug of the electric *lamp*, [illegible]

[illegible] respiratory
[illegible] was transported to hospital, where she died about 14 hours later.

Autopsy

Slight burns of *first and second* grade on the left cheek, left side of the [illegible]
left lower arm [illegible]
on the left s[illegible]
no signs of [illegible]
and in the b[illegible] mucous membrane, which was studded in
several areas with dark *grey to black flakes*. Removal of these flakes exposed an
intense reddening of the underlying membrane.

Microscopic examination *showed* this membrane to consist [illegible]

[illegible]

Stohlman F Discussion of the Reutilization of DNA and DNA Breakdown Products in The Kinetics of Cellular Proliferation p 110 113 New York and London Grune and Stratton 1959

Trowell O A The Lymphocyte Int Review of Cytology 7 235 293 Academic press 1958

Yoffey J M & Courtice C The Lymphocyte in Lymphatics Lymph and Lymphoid Tissue p 391 439 London Edward Arnold Ltd 1956

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REPORT OF CASE

Two women, one aged 72 and [illegible]
cottage. Early one morning the [illegible]
which was about 75 meters away [illegible]
while her neighbours, who had b[illegible]
arrived at most 10 *minutes* late[illegible]
the younger of the two women [illegible] on the floor in the hall before the kitchen. The hall was full *of smoke*. The younger woman lay just before the closed toilet door. They could see flames *in the toilet through* a crack under the door. They carried the woman out into the open air, where she soon recovered and told her neighbours that [illegible]
to the smoke. S[illegible]

[illegible] s a smouldering fire, [illegible] and hardly any *flames*. The lamp [illegible]

[illegible] hours later

Autopsy

[illegible]

[illegible] es exposed an

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ongestion with bleeding out in the alveoli, oedema and, in children, lso emphysema (*Zink, Fischer, Goldschmidt, Schjerning, Mendel*) Bronchitis is rare and when it does occur, it is usually only mild (*Zink*)

In persons who survive a day or so, however, autopsy may show severe acute tracheitis and bronchitis as well as pneumoniae and not infrequently pleural empyema In cases surviving still longer, severe inflammation of the lung tissue may be seen In such patients a reduction in the resistance of the lungs owing to the exposure to the heat is held responsible (*Fischer, Goldschmidt*).

Schjerning appears to be the only one to have suggested that exposure to corrosive smoke and soot might cause pneumoniae In recent literature this possibility appears to have been ignored, and the pneumonia has been ascribed entirely to the exposure to the heat, in accordance with the finding by *Harbitz* that exposure to hot gases can cause necrotic changes in the mucosa with shedding of epithelium and an accumulation of leucocytes at such sites *Harbitz* demonstrated this type of inflammation from the base of the tongue out in the finest branches of the bronchi

Foerster studied persons who had suddenly been exposed to severely hot gases that were forced into the lungs, and he was able to show that in some sites the epithelium projected tuftlike in a uniform eosinofilic substance containing a number of red blood cells and lining the lumina of the bronchi But he could not demonstrate any infiltration of leucocytes or shed epithelium In animal experiments performed under the above mentioned conditions he was able to produce similar changes in the epithelial cells as well as leucocyte infiltration and shed epithelium

Goldbach also studied the effect of heat on the respiratory organs in animals exposed to a hot current of air (600° C) At autopsy he found hyperaemia of the internal organs and, particularly in the lungs, oedema, transudate, bleedings and atelectasis The epithelium of the respiratory tract was shed, the septa of the lungs were swollen with infiltration, particularly of erythrocytes, and in several places the alveolar epithelium had been shed

In the present case necrotizing inflammation was seen in the respiratory tract but not in the mouth or the nose The question that then arises is whether these changes were produced entirely by the heat or whether smoke (as a chemical agent) was also a causal factor Judging from the woman's report, she had not been near the actual fire for more than a second or so Her very slight burns might well be ascribed to the effect of the heat and the fact that no burns were demonstrable in the mouth or the nose also perhaps suggests that she had not inhaled any appreciable amount of hot gas The woman was found lying before the closed door to the room that was on fire, and the air in the room where she was lying was evidently not so very hot, but it was full of smoke It sounds likely that once the woman had realized that she could not put out the fire in the toilet, she had immediately closed



Fig 1
Hamster Trachea after exposure to fire smoke H E × 210

nuclei observed Scattered accumulations of polymorphonuclear leucocytes were found in the deeper layer of the wall of the trachea The lungs showed considerable oedema swelling of the alveolar septa and many of the alveoli contained polymorphonuclear leucocytes The bronchi were filled with leucocyte-containing fibrin and the mucosal epithelium was shed

Autopsy had thus revealed necrotizing inflammation of the trachea and bronchi pneumonia and lung oedema

ANIMAL EXPERIMENTS

Dense smoke produced by burning cork linoleum shavings and dry foliage was conducted for 10 minutes into a cage containing four full grown brown hamsters weighing on the average 100 g The temperature of the smoke varied between 28 and 30° C 24 hours later the animals were killed by exposure to hydrogen sulphide fumes

Soon after the animals had been exposed to the smoke they showed distinct signs of respiratory trouble which persisted until they were killed

Autopsy of the animals revealed a gross reddening and a grey yellow coating of the trachea Histologically the inner wall of the respiratory tract was lined by a thick layer consisting of granular masses which stained red with eosin and densely studded with leucocytes mainly polymorphonuclear (Fig 1) Here and there small groups of shed epithelial cells and even small black granules were seen The surrounding sub epithelial connective tissue contained partly sparse and partly dense accumulations of polymorphonuclear leucocytes The capillaries were widened and in some regions small haemorrhages were seen in the sub epithelial connective tissue These changes could be demonstrated from the larynx down to the middle bronchi though they were most pronounced in the upper part of the trachea The lungs showed capillary stasis

DISCUSSION

In victims dying so soon after a fire accident that any effect of CO can be regarded as negligible the burns are usually so extensive that they can per se explain the death The lung changes usually consist of

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CYTOCHEMICAL DEMONSTRATION OF SOME ENZYME SYSTEMS IN HeLa CELLS[2]

By

PER FORTELIUS, [illegible] and ERKKI SAXÉN

Received 12 viii 60

Cultured cells are steadily gaining the interest of biological research workers They afford excellent material for cytochemical studies, since the handling of preparations can be reduced to a minimum The cells can for instance, be obtained as a monolayer on a coverslip Further, the cell strains are usually cultured in standardized conditions and thus the effect of different factors on the cells can be studied individually The HeLa cell strain, originally cultured by *Gey* (1952) is the most widely used strain everywhere in the world

One possible approach to the problem of alterations in the cells is the comparative study of their enzyme systems, which can be performed even by histochemical methods Of the oxidative enzyme systems succinic dehydrogenase has been demonstrated in HeLa cell cultures by *Kaufman & Hill* (1959), using neotetrazolium as electron acceptor The cells were frozen to minimize unspecific reductase activity and incubated in agar containing medium Sites of succinic dehydrogenase activity appeared as aggregates of formazan crystals in the cytoplasm Alkaline phosphatase, acid phosphatase, unspecific esterase and adenosine triphosphatase have been studied by *Gropp* (1959) and *Gropp et al* (1958 1959), who demonstrated them principally on the cell surface By contrast *Sandritter & Schiemer* (1958), in an extensive study of cytochemistry of HeLa cells, *briefly mention* that alkaline and acid phosphatase unspecific esterase and aminopeptidase are found situated in the cytoplasm unilaterally in the vicinity of the nucleus. Th[illegible]

[illegible] *et al* (1959) have studied alkaline phosphatase with the Gomori method *in alcohol* fixed HeLa cells in connection with other investigations The phosphatase appeared to be distributed diffusely *in the* cytoplasm and to some extent in the nuclei.

[1] Address Maria Hospital Helsinki

[2] This investigation has been supported by grants from the Sigrid Juselius Foundation

the door, but had then inhaled the smoky air a while (at most 10 minutes) in the hall where she lay on arrival of her neighbours

It thus appears less likely that a few minutes' inhalation of the smoky air in the hall, where the smoke was not so very hot, should produce such grave changes in the respiratory organs This assumption is also supported by the fact that no elongated epithelial cells were found anywhere It sounds more likely that the changes had instead been caused, at least in part, by chemical irritation by the smoke inhaled

To check this assumption animals were allowed to inhale dense fire-smoke of about 30° C, which was probably somewhat cooler by the time it reached the animals' cage The results of the experiment strongly support the assumption that the chemical irritation by the smoke is sufficient per se to cause necrotizing inflammation of the airways

It is not known what substances in the smoke are so irritating as to cause inflammation but it is a fact that exposure to sulphur dioxide, for example, or volatile acids forming in association with combustion can produce histologic changes in the airways (*Petri*) closely resembling those seen in the present experiments

SUMMARY

Report of a case of fatal respiratory tract inflammation after exposure to fire smoke

Analysis of the case, autopsy and animal experiments suggested that, in contrast to what is widely believed, such a reaction need not be ascribed entirely to the effect of heat, but can be explained, at least in part, by chemical irritation of the mucosa

LITERATURE

Fischer B & Goldschmidt F Über Veränderungen der Luftwege bei Kampfgasvergiftung und bei Verbrennung Frankf Z Path 23 11 1920

Foerster A Über Veränderungen der Luftröhrenschleimhaut bei Verbrannten Dtsch Z gerichtl Med 19 293 1932

Foerster A Experimentelle Untersuchungen über Veränderungen an den Atmungsorganen bei plötzlicher Einwirkung hoher Temperaturen Dtsch Z gerichtl Med 20 445 1933

Goldbach H J Gibt es vitale Reaktionen der Lunge nach Heissluftеinatmung? Dtsch Z gerichtl Med 45 394 1956

Harb [illegible] (Mordbrand) Vierteljahres

Mend [illegible] hnten Verbrennungen der [illegible] *1870*

Petri [illegible] ie und Histologie Springer

Schje [illegible] Verbrühung vom gerichts[illegible] chtl Med 42 66 1885

Zinl K H Pathologische Anatomie der Verbrennung Veröffentlichungen aus der Konstitutions und Wehrpathologie Bd 10 H 4 5 1940

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particularly in the nucleoli The activity varied greatly from cell to cell in the same culture and seemed to diminish with the aging of the cells

The purpose of the present work was to obtain a general view of some enzyme systems of potential interest in HeLa cells and to develop a series of modification of general histochemical methods which would give satisfactorily uniform results and could be followed routinely in the laboratory This was felt to be a necessary preliminary to a study of the effect of different environmental factors on the enzyme systems of tissue culture cells

MATERIALS AND METHODS

The HeLa cells used were continuously grown in a medium containing 30 per cent of pooled heat inactivated and filtered human serum in Hank s solution Details concerning the culture and properties of this HeLa cell line are to be found in the paper of *Penttinen Saxen et al* (1958)

Succinic Dehydrogenase

After the culture medium was poured off the cells were carefully rinsed twice with saline The substrate solution was prepared according *Nachlas Tsou et al* (1957) except that 0 2 ml of 0 05 M $MgCl_2$ was added to it The cells were incubated in the culture tubes for 2–3 hrs at 37° In some experiments 750 mg of polyvinyl pyrrolidone (MW 11,000 Light) was added in 10 ml of substrate solution Some tubes of these series were frozen in acetone—solid carbon dioxide mixture before incubation In all cases a control preparation was incubated in a medium from which succinate was omitted

Cytochrome Oxidase

The cells were rinsed with saline and incubated at 37° for 1 hr in a medium prepared according to *Burstone* (1959) and containing p amino diphenylamine (Fluka) and 1 hydroxy naphtoic acid 2 (Fluka) The reaction product was chelated with cobalt in 5 per cent neutral formalin rinsed rapidly in water and mounted in glycerine jelly

Leucine Aminopeptidase

The cells were rinsed and incubated at 37° for 2 hrs according to *Nachlas et al* (1958) The copper sulphate used for chelation was dissolved in 5 per cent neutral formalin After fixation and chelation the cells were dehydrated and mounted in Diatex (Bofors) The leucyl β naphthyl amide substrate was prepared by Dajac Laboratories As coupler Fast Blue B salt (*ICI*) was used

AS esterase

Part of the cells were fixed for 5 min in cold neutral formalin after the rinsing [illegible] The method of *Gomori* (1952) was used with [illegible] substrate and diazotized 4 amino 2 5 diethoxy [illegible] coupler The cells were incubated for 2 hrs at room temperature The nuclei were counterstained with carmalum and the cells mounted in glycerine jelly

Alkaline Phosphatase

The cells were rinsed with saline and fixed in cold neutral formalin for 5 mins The reaction for alkaline phosphatase was performed according to *Pearse* (1960) the stable diazotate of 2 benzoylamino 4 methoxy toluidine (Fast Violet B salt ICI) being used as coupler The incubation time was 15 30 minutes at room temperature

Acid Phosphatase

Rinsing and fixation was the same as for alkaline phosphatase. The reaction was made according to Pearse, the stable diazotate of 4 amino 3 1 dimethyl azobenzene (Fast Garnet GBC salt) being used. The incubation time was 1 hr at room temperature.

5-nucleotidase

The rinsed cells were incubated for 2 hrs at 37° in a medium prepared according to the method of *Wachstein & Meisel* (1957).

The main points of the methods are presented in the Table 1 together with some results.

TABLE 1

Enzyme	Pre fixation	Incubation	Post fixation	Localization of activity
Succinic dehydrogenase	–	2 3 hrs at +37° C PVP added	Formol saline	Mitochondria (?)
Cytochrome oxidase	–	1 hr at +37° C	Cobalt formalin	Cytoplasm
Leucyl amino peptidase	–	2 hrs at +37 C°	$CuSO_4$ formalin	Cytoplasm
Naphthol AS esterase	–	2 hrs at room temperature	–	Cytoplasm often unilat near nucleus
Alkaline phosphatase	Neutr formalin 5 mins at +4° C	15–30 mins at room temperature		Cytoplasm
Acid phosphatase	Neutr formalin 5 mins at +4° C	1 hr at room temperature	–	Cytoplasm *often unilat* near nucleus
5 nucleotidase		2 hrs at +37° C	Formalin	Nuclear structures

RESULTS

Succinic Dehydrogenase Fig 1

The activity appeared as seemingly intramitochondrial formazan deposits, evenly distributed throughout the cytoplasm. If polyvinyl pyrrolidone was omitted from the incubation medium the deposits were larger and uneven in size, and the preparations appeared darker than the corresponding ones incubated with polyvinyl pyrrolidone. Controls incubated without substrate were negative in both cases. Freezing and [illegible] esult [illegible] preparation in [illegible] ost giant cells [illegible] greater activity than the normal looking cells.

Cytochrome Oxidase Fig 2

At the sites of activity, brownish black granules appeared fairly evenly distributed in the cytoplasm. In a few cells the granules were

Fig 1

Succinic dehydrogenase Obj × 40 Final enlargement 500 × The activity is localized to coarse granules in the cytoplasm *suggesting mitochondria* A *multinucleated giant* cell with rather pronounced reactivity is seen to the left

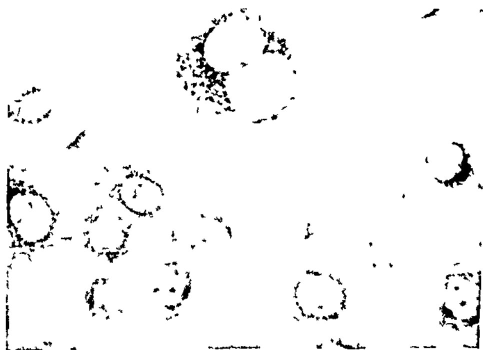


Fig 2

Cytochrome oxidase Obj × 40 Final enlargement 500 × The reaction is strongest around the nucleus especially in a large binucleated cell in the upper part of the photograph To the right is seen a degenerating cell with condensed cytoplasm

situated predominantly unilaterally close to the nucleus In the majority of the cells aggregates of these granules could be seen surrounding the nucleus No reaction however was present in the nucleus itself All the cells of the population seemed fairly evenly stained

Leucine Aminopeptidase Fig 3

The activity appeared as purple or purplish blue granules throughout the cytoplasm The intensity of the reaction varied greatly from cell to cell in the same population Usually it was most intense in the cells growing in the middle of the coverslip Some giant cells showed more activity than the normal sized cells and some less

AS Esterase Fig 4

A bright blue finely granular precipitate indicated the activity of esterase The reaction in the cells seemed to be most intense in the cytoplasm unilaterally in the vicinity of the nucleus a fact which is suggestive of the Golgi apparatus (*Sandritter* 1958) The nuclei were completely inactive which enabled them to be counterstained with carmalum The reaction was usually rather uniformly distributed among the living cells of the population The giant cells however appeared to be more active than the cells of normal size

Fixation with formalin caused diminution of the intensity of the reaction and did not improve distinctness of the localization

Alkaline Phosphatase Fig 5

A brown or brownish black precipitate formed at the sites of activity in the cytoplasm The activity was generally strongest around the n[illegible]

[illegible] preparations often appeared patchy The patchiness could be avoided however to a certain degree by rapid fixation with formalin which does not diminish the activity In seemingly homogeneous preparations the activity of different cells varied The giant cells very often showed a low activity

Acid Phosphatase Fig 6

The activity appeared very like that of alkaline phosphatase but was on the whole more evenly distributed The reddish brown granules were mainly situated unilaterally close to the nuclei in the cytoplasm In giant cells a distinctly higher activity could be seen Formalin fixation was found to give good results

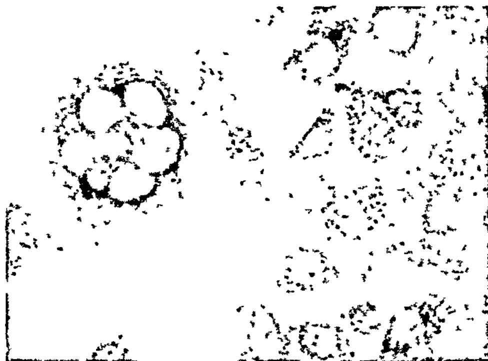


Fig 1

Succinic dehydrogenase Obj × 40 Final enlargement 500 × The activity is localized to coarse granules in the cytoplasm suggesting mitochondria A multinucleated giant cell with rather pronounced reactivity is seen to the left



Fig 2

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sults



Fig 3
Leucine aminopeptidase Obj × 40 Final enlargement 500 × The enzyme activity is evenly distributed in the cytoplasm The contrast seen in many nucleoli is presumably due to an unspecific reaction

5'-nucleotidase Figs 7 a and b

A positive reaction was indicated by brown deposits almost entirely restricted to the nuclei, the nucleoli being most intensely stained In dividing cells only the chromosomes seemed to be coloured In giant cells some activity was present even in the cytoplasm

DISCUSSION

During these studies we have noted that it is possible to handle cell cultures with the routine histochemical methods When the rinsing of the cell cultures in the tubes and pouring of reagent solutions is carefully done, practically all the cells are preserved The observations concerning the effect of fixation are compatible with the information Pearse gives about the tissue enzymes in his book The cells can be fixed with cold neutral formalin before demonstration of phosphatases, this is even preferable The AS-esterase activity, however, is diminished by fixation Succinic dehydrogenase, cytochrome oxidase leucine amino peptidase and 5'-nucleotidase do not tolerate even a short fixation

The observed localization of succinic dehydrogenase very probably corresponds to the mitochondria When these were osmotically protected with polyvinyl pyrrolidone (*Scarpelli* 1958), the formazan deposits were quite uniform in size and intensity of colour Without protection, the deposits were variable in size due to swelling and possible

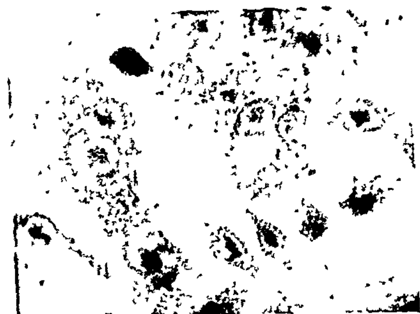


Fig. 4

Naphthol AS esterase. Obj. × 40. Final enlargement 500 ×. To the left a giant cell exhibiting a strong reaction is seen. In some other cells unilateral juxtanuclear activity may be observed. Above the giant cell is situated a condensed cell. The prominence of the nuclear structures depends upon counter staining with carmalum.

rupture of the mitochondria, which even causes an increase in the intensity of the reaction. In osmotically protected cells preparations the most active cells (giant cells) apparently have a greater density of *mitochondria*.

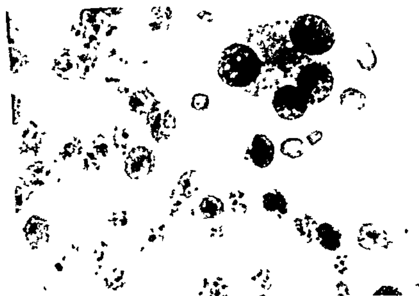
The localization of leucine aminopeptidase seems not to be connected with any cytoplasmic structure. The dye granules formed are relatively evenly scattered in the cytoplasm with a slight tendency to concentrate unilaterally close to the nucleus. This is not quite in agreement with the observations of *Sandritter* (1958), who claims that aminopeptidase is present predominantly in the region corresponding to the Golgi apparatus. Whether the deviation between his results and ours is due to differences in technique cannot be decided, since no details of the method used were given in his article. In this connection it should also be pointed out that HeLa cell strains used in different laboratories may differ very much from each other.

The activity of acid phosphatase and AS esterase, however, is greatest in the vicinity of the nuclei unilaterally, so that the reaction product forms a kind of pointed cap on one side. This probably indicates the site of the Golgi apparatus as suggested by Sandritter. Using Naphthol AS acetate substrate no reaction on the surface of the cell could be demonstrated. This is in contrast to the results obtained by *Gropp*

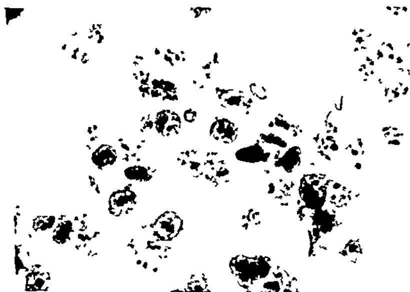
Fig 5
Alkaline phosphatase Objective × 40 Final enlargement 500 × The reaction typically varies from cell to cell Giant cells with low activity are seen in the middle and to the right



Fig 6
Acid phosphatase Obj × 40 Final enlargement 500 × The activity in the giant cell is very marked In the upper left part a condensed degenerating cell is seen In some cells unilateral juxtanuclear activity is visible



Figs 7 a and b
5 nucleotidase Obj × 40 Final enlargement 500 ×
a Localization in nuclear structures In the giant cell a specific reaction is also encountered in the cytoplasm In the other cells black apparently nonspecific deposits of lead sulfide appear



b Somewhat above and to the left of the middle is a cell undergoing mitotic division The chromosomes exhibit strong reactivity

Fig 5

All aline phosp1 atase Ol jective × 40 Final enlargement 500 × The reaction typ c ally varies from cell to cell Giant cells with low activity are seen in the m ddle and to the right



Fig 6

Aci l p h p1 atase Ol j × 40 Final enlargem nt 500 × The activity in tl e g ant cell is very marke l In the u per left part a c nden ed degenerating cell s seen In some cells unilateral juxtan iclear activity is v sil le

Gropp A Vergleichende enzymhistochemische Beobachtungen zum Phosphatase

Grop

15 209 1958

Gropp A & Hellweg H R Cytologische und enzymcytochemische Untersuchungen uber die Zelloberflache an *in vitro* Gezuchteten Zellen Ztschr f Zellforsch 50 315, 1959

Kaufman V & Hill R Determination of Succinic Dehydrogenase Activity in Tissue

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Wachstein M & Meisel F Histochemistry of Hepatic Phosphatases at a Physiologic pH with Special Reference to the Demonstration of Bile Canaliculi Am J Clin Path 27 13, 1957

(1959), who used α-naphthyl acetate as substrate and found the activity predominantly on the surface.

The reaction for alkaline phosphatase, too, seems to be most intense round the nucleus, having a tendency to unilaterality, although never as much as acid phosphatase and esterase.

5'-nucleotidase differs from all other enzymes studied in this work. Its activity is almost entirely restricted to the nucleus, particularly to the nucleoli and apparently to the chromatin structures. In mitotic cells the activity can be seen in the chromosomes. Only the giant cells exhibit activity *in the cytoplasm, where it is unilaterally distributed close* to the nucleus.

In all preparations some degenerating cells could be seen. These appeared darker than any other cells in all the enzyme reactions studied. This phenomenon is partly due to shrinking of the cytoplasm and subsequent condensation of the cell and probably does not indicate any real increase of activity. In giant cells the increased or decreased activity *is real and indicates deviations in the metabolism*.

The differences noted in this work between the enzyme patterns of the ordinary HeLa cells and the giant cells suggest a possibility for detection of metabolically differentiated cells of the same strain, assuming that the experiments are made with a sufficient number of controls. Some pilot tests made are quite encouraging (*Fortelius et al.* 1960).

SUMMARY

Succinic dehydrogenase, cytochrome oxidase, leucine aminopeptidase, AS-esterase, alkaline and acid phosphatases and 5'-nucleotidase have been demonstrated in HeLa cells.

Succinic dehydrogenase is present in the mitochondria in the cytoplasm. The localization of acid phosphatase, AS-esterase and to a certain degree even alkaline phosphatase and cytochrome oxidase, is most intense in the cytoplasm in the vicinity of the nucleus, a fact suggestive of the Golgi apparatus. Leucine aminopeptidase is evenly distributed throughout the cytoplasm. 5'-nucleotidase is present only in the nucleus, especially in the nucleoli of ordinary cells. In giant cells even the cytoplasm shows activity.

REFERENCES

Bmes C., Planel H. & David J. J.: Reserches histochimique sur la souche cancéreuse HeLa. C. R. Soc. Biol. (Paris) *153*: 347, 1959.

Burstone M.: New Histochemical Techniques for the Demonstration of Tissue Oxidase (Cytochrome Oxidase). J. Histochem. Cytochem. *7*: 112, 1959.

Fortelius P., Saksela E. & Saxén E.: A HeLa Cell Clonal Line Lacking Alkaline Phosphatase Activity. Exptl. Cell Res. *21*: 616, 1960.

Gey G. O., Coffman W. D. & Kubicek M. T.: Tissue Culture Studies of the Proliferative Capacity of Cervical Carcinoma and Normal Epithelium. Cancer Res. *12*: 264, 1952.

Gomori G.: The Histochemistry of Esterases. Int. Rev. Cytol. *1*: 323, 1952.

Gropp A Vergleichende enzymhistochemische Beobachtungen zum Phosphatase nachweis an einigen Carcinomzellstammen *in vitro* sowie uber milieubedingte Veranderungen der Enzymaktivitat Ztschr f Krebsforsch 63 156 1959

Gropp A Bontke F & Hupe K Untersuchungen zum Nachweis der Alkalischer Phosphatase an *in vitro* gezüchteten menschlichen Carcinomzellen mit besonderer Berucksichtigung ihrer Beziehung zu der Zelloberflache Exptl Cell Res 15 209 1958

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Scarpelli D G & Pearse A G E Physical and Chemical Protection of Cell Constituents and the Precise Localization of Enzymes J Histochem Cytochem 6 369 1958

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HAEMOPHILUS VAGINALIS AND ITS ROLE IN VAGINITIS

By

S P Lapage[1]

Received 24 viii 60

Several papers have recently been published suggesting that many cases of vaginitis are due to small Gram-negative rods, and claims have been put forward for the recognition of a new species called *Haemophilus vaginalis* However, the reports on the characters of the organisms which have been considered *H vaginalis* differ in some important ways among themselves A general summary of the described characters of *H vaginalis* and of the clinical condition, is given below Table 1 lists some of the characters described by various authors Tables 2A and 2B list the incidence of *H vaginalis*, the type of cases investigated and the diagnostic criteria

H vaginalis is a Gram-negative, small, pleomorphic rod Cocco-bacillary forms are common It is fastidious in its growth requirements, and the best medium for isolation is Casman's blood agar (*Casman* 1947), or a similar medium Incubation with added carbon dioxide aids the isolation The colonies are small at 48 hours, and the organism may or may not lyse the blood The organism shows some biochemical activity and ferments some sugars, producing acid The reports of its sensitivity to antibiotics vary considerably It shows little or no virulence for small laboratory animals

Infection may be symptomless, or may be accompanied by a mild vaginitis *H vaginalis* is reported to disappear when treatment is suc-

[1] The author is now at the Department of Bacteriology University College Ibadan Nigeria

This work was carried out at the State Serum Institute Copenhagen under a Carlsberg Wellcome Travelling Research Fellowship and a further grant from the Carlsberg Foundation to the State Serum Institute to continue the work

I wish to thank Dr *J Ørskov* and the staff of the State Serum Institute for their help and kindness especially Dr *A Reyn* in whose department the work was done Drs *H Lautrop* and *B Korner* for their help and advice and Miss *Aase Langhorn* and her staff who so patiently prepared the media My thanks are also due to Drs *H G Bertelsen Find Andersen* and *G Stakemann* for the specimens and clinical details

Dr *S T Cowan* of the National Collection of Type Cultures London kindly supplied strains of *Haemophilus spp* Professor *C R Amies* of the University of Alberta and Dr *A Eyquem* of L'Institut Pasteur Paris strains of *H vaginalis* and Mr *J Szabo* of Lundbeck Pharmaceuticals Denmark the strain of *H vaginalis* from Drs *Gardner & Dukes*

cessful Human volunteer experiments are suggestive, though more experimental infections were obtained when discharge was inoculated vaginally than when pure cultures were used (*Gardner & Dukes* 1955)

TABLE 1

Some of the Characteristics of H vaginalis as Described by Other Authors

	Leopold 1953	Gardner & Dukes 1955	Worch & Lutz 1955	Amies & Jones 1957
Gram stain	—	—	— or variable	—
Pleomorphism	Some	Some	Some	Marked
Colony size	Small	Small	Small	Larger
Lysis	β	Vary	Greening & β	—
Oxidase	—	—	NS	—
Nitrate	—	NS	—	+
O_2 need	Microaerophil	Microaerophil	NS	Aerobic
Improved CO_2	NS	+	—	—
Glucose	A	A	A	A
Lactose	—	—	—	—
Various other sugars tested with some variable results				
Penicillin	NS	R	S	S or R
Streptomycin	NS	R	S or R	S
Chloramphenicol	NS	R (S)	S	S
Tetracyclines	NS	S	S	S
Bacitracin	NS	S	S	NS

NS = not stated in report

The reported incidence of the organism in cases of vaginitis varied from about 5 50 per cent, and in one series it was not found in any case They are given in Tables 2 A and 2 B (*Doll* 1958) It was common to find it associated with *Trichomonas vaginalis* and *Candida albicans*

Diagnosis was made on clinical grounds, on microscopical examination of stained and wet films of the discharge, and on culture of the organism in most cases However, in some of the reports, diagnosis was made without culture According to *Gardner & Dukes* (1955), the organism was often the only one found on the isolation plates but most of the other authors do not regard this as common

The stained films showed numerous small Gram negative pleomorphic rods Epithelial cells were plentiful in the wet mounts often showing a refractile cytoplasm due to the bacterial masses on them *Gardner* & Dukes (1955) called this the "clue cell" The other authors have remarked that such cells were common and diagnostic in the specimens Leucocytes were considered scanty by most authors, although *Amies & Jones* (1957) found them *frequent*

Leopold (1953) was the first to describe the isolation of such an organism and has summarized its characters in a more recent symposium (*Leopold* 1956) He isolated it from the urines of men and from swabs of women with cervicitis All of the men in whom it was found had prostatitis with or without urethritis *Gardner & Dukes* (1954 1955) isolated the organism from cases of vaginitis and

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methods of *Gardner & Dukes Brett & Cohen Debray* (1959) stress the necessity for for a suitable transport medium a rich medium for growth and incubation in carbon dioxide

TABLE 2B

Distributions of H vaginalis in Male Patients

Author	Total cases	Type of case	% H vaginalis isolated	Diagnostic criteria
Leopold 1953	965	Unknown All positive cases had prostatitis +/— urethritis	5 5	Cult urines
	9	Husbands of wives with positive cultures	44 5	Cult urines
	18	History non specific urethritis	11 1	Cult urines
Gardner & Dukes 1955	6	Husbands of wives with recurrent infections	100	Cult urethral
	47	Husbands of wives with *H vaginalis* infections	96	Cult urethral
	?	Husbands after experimental inoculation of wives	3 cases	Cult urethral
Ray & Maughan 1956	12	Wives infected and resistant to treatment	50	Smear urethral
Amies & Jones 1957	371	Non gonococcal urethritis	8	Smear urethral

MATERIAL

Group I

Preliminary work was done on several hundred routin[illegible] plates These [illegible] and incubated [illegible]

(described below), as in [illegible] selection

Group II

Smears and cultures from 156 patients were examined all in the childbearing period of life [illegible] They were divided into [illegible] who filled in a printed [illegible] clinical state [illegible] ermine the determin[illegible] were nor[illegible] many w[illegible] percenta[illegible]

The following criteria were used for classification -

from some of the husbands of these cases They named the organism *Haemophilus vaginalis* and described the clue cell and clinical condition in a survey of just under 1 200 patients They reported and discussed the incidence of infections with *T vaginalis C albicans H vaginalis* and other pathogens, and the results of volunteer experiments *Gardner Dampeer & Dukes* (1957) described a similar survey of over 3 000 private and clinic patients

TABLE 2A

Distribution of H vaginalis in Female Patients

Author	No of cases	Type of case	% H vaginalis isolated *	Diagnostic criteria
Leopold 1953	58	Cervicitis +/—erosion	27	C
Gardner & Dukes 1955	291	Private obstet & gynae	48 5	W M S C
	43	Other conditions e g cervicitis	0	
	78	Normal controls	0	
Gardner, Dampeer & Dukes 1957	1211	Vaginitis	44	W M S C
Wurch & Lutz 1955	500	Leucorrhoea	22	S C
Lutz & Wurch 1958	100	Normal pregnancy	20	
	126	Trichomonas cases	28 5	
Ray & Maughan 1956	231	Clinic obstet & Gynae	39	W M S
	75	Pregnancy & vaginitis	40	
Amies & Jones 1957	371	Cases of cervicitis	5 1	W M,S C
	829	Grade III cervical smears	41 positive smears	S only available
Brewer et al 1957	211	Leucorrhoea	42	W M S+/ C
Doll 1958	300	Vaginitis	0	W M S C

* The percentages are difficult to derive in some of these reports but have been worked out from the available data W M – Wet Mount S Stained smear C Culture

Lutz & Wurch (1954) had noticed the common occurrence of small Gram negative rods in the vaginal flora during pregnancy Later (*Wurch & Lutz* 1955 *Lutz et al* 1956 a b 1957) they reported further study of these organisms and of organisms from cases of leucorrhoea Their clinical description agrees substantially with that of *Gardner & Dukes* (1955) They named their organism *Haemophilus vaginalis haemolyticus* and stressed its frequent occurrence with *T vaginalis Ray & Maughan* (1956) described a series of cases but based their diagnosis on clinical grounds and wet mounts and smears alone *Brewer et al* (1957) described a series of cases using clinical microscopical and sometimes cultural grounds for diagnosis *Amies & Jones* (1957) investigated cases of cervicitis and found a much lower incidence of their organisms (Tables 2A and 2B) and rather different characters (Table 1) They used a serum yeast medium for isolation unlike the other authors who all used blood

and a lithium chloride lauryl sulphate chocolate agar (for recipes, see below) With most of the specimens, none of the selective media offered any advantage over careful examination of a well spread Casman's medium plate The plates were incubated at 37° C for 48 hours with approximately 8 per cent carbon dioxide and a moist atmosphere in a closed jar

The plates were examined at 48 hours and films made of all the various type of

18 24 hours and then films were prepared and stained by Gram's method The plates were kept for a further 4 5 days at 37° C without added carbon dioxide, and examined for any new type of colony, and further differentiation of the original ones

All the small rods proving Gram negative deeply staining or Gram variable and some Gram positive rods, for purposes of comparison were kept and freeze dried They were also subcultured on to a nutrient agar plate with a "grey air coccus" streaked across it Gram negative rods, obviously not of this series, which grew on nutrient agar were rejected Such rods included coliforms and a few miscellaneous organisms such as *B anitratum, Pseudomonas spp* etc

Media used –

5 0 g, yeast extract
thioglycollate 0 5 g.
for 20 minutes Dis-
and plug with wool plugs
impregnated with paraffin wax

Casman's medium (*Casman* 1947) (Original formula) Bacto proteose peptone

a mixture 25 per cent blood and 75 per cent water) The latter may be omitted if the blood is slightly lysed by storage

Gardner & Dukes modified this slightly by adjusting to pH 7 5 and using 5 per cent rabbit blood Their method was followed in this investigati

defibrinated horse blood (6 7 per cent) and mix Heat in a water bath at 80° C for a few minutes, to chocolate the medium Cool to 56° C in a waterbath

Add 300 ml of sterile ascitic fluid which has been warmed to 56° C Pour plates

heating in a bath at 80° C

Condition	No of cases	Obligatory Symptom (Subjective)	Obligatory Signs (Objective)
Vaginitis	30	Discharge	Inflammation of vaginal wall redness oedema etc
Cervicitis	20	*Discharge*	Visible cervical lesion
Vaginitis and Cervicitis	8	Discharge	Signs of both
Discharge	20	Discharge	Discharge seen
Miscellaneous	6	All had Discharge	Miscellaneous--for example fibroma of uterus
Normal	32	None	No abnormality of genital tract detected

A few of the patients with vaginitis with cervicitis and with both had adnexitis as well

The series consisted of primary specimens only Follow up specimens were not included No cases in this series had recent treatment In 2 4 per cent of the cases the plates were overgrown with *Proteus spp* these cases were removed from the series The specimens were not examined for obligate anaerobes or *Mycoplasma spp* Yeasts were *of low incidence (3 Torulopsis glabrata 2 C albicans)* but no special medium was used for their isolation *T vaginalis* was looked for in wet mounts from many of the hospital patients but the number of cases was too small to relate statistically either to the clinical condition or the incidence of Gram negative rods However the cases in which *T vaginalis* or yeasts were found have been retained in the series and therefore it is possible that they were the prime cause of some of the cases of vaginitis

The pH was determined by the clinicians using pH papers but they found that the method was difficult to apply and its use was discontinued

PROCEDURE

Films – Two smears of the discharge were made using an ordinary sterile swab and stained one by Gram s method and one with Leishman s or Giemsa s stain They were examined for cells and organisms

Wet mounts were not examined for clue cells largely because staining appeared *necessary to determine what kind of organisms were on the cells* It is possible that clue cells in stained films showed a different incidence from clue cells in wet mounts *the method used by some of the other authors* and that artefacts were created in staining In some cases clue cells appeared to be due to the large number of bacteria on the films so that many organisms coated the cells but often when there were large numbers of organisms due to the process of drying they tended to be massed round the edges of the cell rather than on it causing an appearance differing from that of a clue cell In other cases it seemed that there were clumps of organisms on some of the epithelial cells while the film as a whole had a lower [illegible] *to some physical attraction by the* [illegible] ree types of clue cell were found [illegible] (true clue cell) those with small Gram negative rods mixed with other organisms (mixed clue cell) and those with other organisms only (false clue cell)

Cultures Swabs were taken from the posterior fornix with sterile precautions using a buffered charcoal impregnated swab (*Stuart et al* 1954) They were placed immediately in a thioglycollate transport medium which was prepared freshly once a week but with no resazurin or methylene blue in it They always reached the laboratory within *four hours of having been taken often within a shorter time*

They were plated onto Casman s blood agar (Casman s Medium) with and without a penicillin tablet Casman s medium with 1/500 000 crystal violet a chocolate agar with added ascitic fluid (Modified McLeod s Medium) (*McLeod et al* 1934)

and a lithium chloride lauryl sulphate chocolate agar (for recipes see below) With most of the specimens none of the selective media offered any advantage over careful examination of a well spread Casman s medium plate The plates were incubated at 37° C for 48 hours with approximately 8 per cent carbon dioxide and a moist atmosphere in a closed jar

The plates were examined at 48 hours and films made of all the various type of c lony on the plate not only those conforming to the descriptions of *H vaginalis* and stained by Gram s method Subcultures on to Casman s medium were made from single colonies of all Gram negative small rods and all suspicious Gram positive or Gram variable rods Any type of colony which was too sparse or too small to film was subcultured The subcultures were incubated with added carbon dioxide for 18 24 hours and then films were prepared and stained by Gram s method The plates were kept for a further 4 5 days at 37° C without added carbon dioxide and examined for any new type of colony and further differentiation of the original ones

All the small rods proving Gram negative deeply staining or Gram variable and some Gram positive rods for purposes of comparison were kept and freeze dried They were also subcultured on to a nutrient agar plate with a grey air coccus" streaked across it Gram negative rods obviously not of this series which grew on nutrient agar were rejected Such rods included coliforms and a few miscellaneous organisms such as *B anitratum Pseudomonas spp* etc

Media used –

Thioglycollate transport medium

Pancreatic digest of casein BBL Trypticase 15 0 g dextrose 5 0 g yeast extract

... paraffin wax

Casman s medium (Casman 1947) (Original formula) Bacto proteose peptone

Add 300 ml of sterile ascitic fluid which has been warmed to 50° C Pour plates

Lithium Chloride Lauryl sulphate Medium

Sod

Pou

from their 24-hour subculture It is possible that these cultures were mixed but the shape and size of the cells of any strain remained constant, the strains were filmed *many times, and in some of the cases serial single colony isolations were carried out*

The films were made from 18 hour old cultures from Casman's medium plates and the staining process was controlled with a gonococcus and a pneumococcus The method of Gram staining was with carbol methyl violet and carbol fuchsin and the constituents and method of preparation of the stains is given in *Kristensen* 1953 *Decolourization was carried out with 99 per cent* alcohol applied for 2 minutes

Types of the Small Colony Found, and Their Nature

Many of the small colonies found on the plates were circular, entire, shiny and melted on touching The majority showed no special differential characters, but some were flatter or matt or distinguishable in other ways Many caused slight greening of the blood medium round the colonies Lysis was unusual and nearly always *from* lytic streptococci or larger colonies such as micrococci or coliform organisms The small Gram-negative rods did not seem to produce a colony easily distinguishable from those of many of the small Gram-positive strains α- or γ-lytic streptococcal colonies were less easily confused

Many of the authors described "melting on touch" as characteristic of *H vaginalis* This property is common to many of the small 24 48 hour colonies of both Gram-positive and Gram-negative strains, although, when older, the colonies of both types may be soft, but do not melt on touching In general, when a 24–48 hour colony, or part of it, could be moved about entire on the plate, then it did not consist of small Gram-negative rods

Strains of both Gram-negative and Gram-positive small rods, and of many other organisms, caused greening of the blood round them to a small extent, but only streptococci and some Gram-positive rods caused greening of modified McLeod's medium

Most of these small colonies consisted of small Gram-positive rods which would be described as "diphtheroids", many showing pallisading, snapping division, clubbing, barred forms, and other such characteristics Microscopically, the Gram-positive and Gram-negative small rods often resembled each other in size and shape, though frank pallisading was commoner with the Gram-positive, and clubbing and barring were useful differential characters Large Gram-positive rods of the lactobacillary type were also found, though their colonies were often minute when grown aerobically on Casman's or modified McLeod's medium

The organisms, in general, were very delicate, and needed subculturing once a day on solid media to be certain that strains were not lost A heavy inoculum was preferable They were sensitive to different batches of media, and a few strains might be lost suddenly, for no apparent reason Eight of the thirty three strains were lost but as many of their properties had been determined, and as films of them were available, they have been classified and included in the incidence of the strains On several occasions, strains were only regained from the freeze-dried cultures

BACTERIOLOGICAL INVESTIGATION

Source of Strains

54 strains of small Gram negative rods were examined in detail 22 from the patients in Group II 15 from the patients in Group I and 17 stock strains (3 *H influenzae* 1 *H para influenzae* 1 *H suis*, 1 *H canis* and 11 strains of *H vaginalis* (one isolated by *Gardner & Dukes* and 10 isolated by *Amies & Jones*)) In addition 4 small Gram positive rods were examined in detail for comparison 26 were examined for their antibiotic sensitivity only, owing to the differing reports on the antibiotic sensitivity of *H vaginalis*

METHODS

... per cent haem... 1 per cent yeast extract and 0 2?5 per cent Japanese agar added This will be referred to as "semi solid Casman s medium)

Colonial morphology amount of growth and *haemolysis* were examined on Casman s medium (rabbit blood) Four main types of effect on the blood were seen (i) the strain did not affect the blood at 24 hours but at 48 hours there might be slight greening where the growth was heavy but not round isolated colonies No change occurred on further incubation (ii) there was some greening of the blood at 24 hours round the ...

... colonies (iv) β lysis

Presence of growth was tested on Fildes peptic blood extract agar (*Mackie & McCartney* 1953 p 190 ...
19 5
and
colo ... *Satellitism*
was ... streaking a ... grey ...

... a closed jar Such a jar ... growth by and need for

... the ... strains which failed to

Oxidase was tested for by rubbing a little growth from a modified McLeod's plate on to a filter paper on which a solution of tetra-methyl phenylene diamine hydrochloride had been placed

Nitrate reduction was tested on Casman's medium by inoculation into the medium near a piece of filter paper, soaked in 40 per cent KNO_3 and dried laid on the plate (*Cook* 1950). The plates were incubated aerobically. Anaerobic incubation may be more successful for *Haemophilus spp* Areas of greening of 0 5 2 cm diameter or more were shown by the positive strains Some of the nitrate negative strains caused greening of the blood for a millimetre or so round the stab when a nitrate paper was not present on the medium and possibly a very weak nitrate reduction could have been masked *H_2S production* was tested for with lead acetate papers above semi-solid Casman's medium *Indole production* was tested for by inserting an oxalic acid paper above semi-solid Casman's medium without glucose *Sugar reactions* were tested with semi-solid Casman's medium without glucose or starch but with bovine albumin to a final concentration of 0 5 per cent, and 0 0003 per cent Haemin 1 per cent yeast extract and 0 225 per cent agar added

Antibiotic sensitivity was tested on Casman's medium, using high concentration tablets (dihydrostreptomycin 3 mg, aureomycin 1 mg, chloramphenicol 1 mg, bacitracin 10 mg) and measuring the zones of inhibition (*Lund* 1951, 1955) Penicillin was similarly tested using a tablet containing 2 5 i u *Sulphathiazole* (10 mg) was tested on a peptone-free chocolate horse blood placenta broth agar with added ascitic fluid

RESULTS

The organisms could be divided into the following 5 types

(a) 23 *H influenzae* type (and 2 atypical strains and 1 *H canis*)
(b) 22 possible *H vaginalis*
(c) 3 *miscellaneous small Gram-negative rods*
(d) 3 *Gram-negative to Gram-positive rods*
(e) *Frankly Gram-positive small rods*

The differentiating characters of the types are presented in Table 3 and discussed in the large print below Other characters of less diagnostic value are given in small print, but may be just as important from other points of view If, in any type, the number of strains is not given for any tests, it can be assumed that all were tested

(a) 23 *H Influenzae Type*

This type comprised 3 stock *H influenzae*, 1 *H para-influenzae*, 1 *H suis*, 9 of the 10 *H vaginalis* strains from *Amies & Jones*, and 9 of the 16 isolates from Group I

2 atypical strains and *H canis* are mentioned here They resembled this type more than any other type They are described below

These morphologically resembled the stock *H influenzae* strains Pleomorphism was pronounced, filaments were often present, the cells were bacillary or cocco bacillary and were uniformly Gram-negative

Their 24-hour colonies on Casman's medium were circular, entire or slightly irregularly edged shiny, transparent flat domes up to 0 5 mm which melted on touching At 48 hours they became colourless, transparent, delicate colonies with regular or slightly irregular edges with

raised centres, and were up to 1.5 mm in diameter. Later they increased in size up to 2–3 mm, the colonies differentiated showing raised centres and irregular edges, giving a poached egg type of appearance. This was the general appearance, although minor variations occurred, and some of both *Amies & Jones'* strains and of our isolates and *H suis* gave smaller colonies.

TABLE 3

Some Characteristics of the Strains Investigated

	H influenzae type	Possible *H vaginalis*	Miscellaneous Gram negative group	Gram negative to Gram positive group	Small Gram positive rods	Small Gram positive rods
Number	23	22	3	3	4	26
Gram stain*	—	—/V	—	—/V/+	+	
Morphology†	H_I	H_V (D)	D	D	D	
Amount of growth & colony size	++	+	+	—	—	
Colony and type†	H_I	H_V	H_V or D	D or H_V	H_V or D	
Effect on rabbit blood (plate)§	none	G	G or none	G or none	G or none	
Growth on Casman's without blood	—	+	+	—(+)	+	
Growth on Fildes & Levinthal's	+	—	—	—	—	
Satellitism	+	—	—			
Catalase	+	—		—		
Oxidase	+		—	—		
Nitrate	+		—	—	—	
Penicillin°	R or S	S	S	S	S	S(r)
Bacitracin	R	S	S	S	S	S(r)
Streptomycin	S	S	S	S	S	S(r)
Chloramphenicol	S	S	S	S	S	S
Aureomycin	S	S	S	S	S	S(r)

* — = Gram negative V = Gram variable + = Gram positive
† H_I = *H influenzae* H_V = *as H vaginalis type* D = Different
§ G = greening (see text)
— = one strain showed it slightly
° R = resistant S = sensitive S(r) *Some resistance shown by an occasional strain*

The characters of this type *not shown in Table 3 may be summarized* as follows. The growth on Casman's medium was fair in amount but more on modified McLeod's medium, single colonies showed no effect on ... β-lytic strains of ... in this series). The ... They grew on ... factor only (the stock *H para influenzae* and 1 *strain from Amies & Jones*). Growth was not improved by 8 *per cent* CO_2 on Casman's or modified McLeod's media. They showed the aerobic type of growth in thioglycollate, they were insensitive to phenol red (10 strains only tested), indole was not produced except by the 3 stock *H influenzae* and 2 of *Amies & Jones'*

strains and H_2S was not produced, except by the stock *H para-influenzae*, the *H. para-influenzae* like strain from *Ames & Jones* and 1 *H influenzae* type (weak production was shown by these strains)

The 2 atypical strains Both these resembled the *H influenzae* type more than they resembled any other type One was a strain of *H vaginalis* isolated by *Ames & Jones* It was a Gram-negative rod consisting of long, fine filamentous cells in an entangled hair pattern The colonies were flatter and larger than the *H influenzae* type and became radially striated after a few days It was β-lytic on rabbit blood, and produced a soluble lysin, and was oxidase negative It did not reduce nitrates It produced H_2S strongly

The other was a strain from Group II This strain was a tiny Gram-variable rod which formed small, dry and coherent colonies, which differentiated after a few days and developed protruberances and resembled a circular half bunch of grapes The organism was non-lytic on Casman's medium, but lysed the blood slightly in the soluble lysin test It was oxidase negative, did not reduce nitrates and was a strong H_2S producer It was also relatively resistant to aureomycin

In all other characteristics tested, these two strains resembled the *H influenzae* type

H canis differed also Its colonies were larger, denser and of stiffer consistency It needed X and not V factor

These two strains and *H canis* will not be discussed further

(b) *22 possible H vaginalis*

This type comprised the *H vaginalis* stock strain from *Gardner & Dukes*, 6 strains from Group I, and 15 strains from Group II 7 of these strains resembled the others in all but morphology, and their growth in the thioglycollate medium They are included in this type, and the differences are discussed below

They were Gram-negative or deeply staining They were more deeply staining than *H influenzae* and needed carefully controlled staining to determine whether they were Gram-positive or Gram-negative They were small rods, often in pairs, approximately 0 2 to 0 4 × 1 0 μ, or sometimes more cocco-bacillary Filaments were not seen Pleomorphism was not striking and was less than *H influenzae* The cells showed a typical arrangement differing from *H influenzae* Bizarre and degenerate forms were rare, even in 48-hour cultures

Some of the strains were sharper in outline, and less coccoid, with squarer ends They sometimes showed more pallisading and sharper angles between cells than the stock strain Occasionally they developed granules at the ends which did not cause bulging of the cells Morphologically, this type of rod is only distinguishable by the staining reaction from strains of small rods with Gram-positive granules, whose whole cell becomes Gram-positive on subculture, and from certain frankly Gram-positive rods

The seven strains previously mentioned usually resembled the *H vaginalis* type on films from the isolation plates, but after subculturing, and especially on films of 48-hour cultures, they showed a more boat-shaped, rather irregular looking cell, which stained unevenly, giving the mass of cells a blotchy appearance Individual cells also appeared more pleomorphic and degenerate Strains were found which could be placed in a series showing all gradations from these to Gram-positive forms where an uneven Gram-positivity of the cell gave a similar blotchy appearance

24 hour colonies on Casman's medium were circular, smooth domes with entire edges and shiny surfaces, up to 0 3 mm in diameter They melted on touching At 48 hours they were still smooth domes and grew up to 0 75 mm, with a whitish tinge to the colony The mass of growth was whitish grey and the colonies appeared whiter and denser than the *H influenzae* type Later, no further differentiation occurred as a rule, though in some of the strains the colonies developed a slight point, giving the colony a low conical appearance Individual colonies may reach up to 1–1 5 mm, and, in some strains, both the mass of the growth and individual colonies developed a slight metallic sheen The strains showing the blotchy type of staining had colonies resembling the *H vaginalis* type though in some strains they were a little smaller

The characters of the possible *H vaginalis* strains not in Table 3 are as follows

The growth on Casman's medium was less than *H influenzae* and even poorer on modified McLeod s medium Single colonies caused greening of rabbit blood in 24–48 hours and some strains, on further incubation, turned the whole plate chocolate and produced clear zones round single colonies Soluble lysin was not produced They did not grow or very poorly, on ascitic fluid agar (results varied from batch to batch) They did not appear to need X and V factors but showed inhibition by toxic factors in the medium They were sensitive to phenol red (only 4 strains tested), growth was improved by 8 per cent CO_2 on modified McLeod s medium, more or less indifferent on Casman's medium (one strains needed CO_2 to grow on modified McLeod's medium) In thioglycollate media 17 showed aerobic and 6 microaerophilic types of growth Indole and H_2S were not produced

(c) *3 Miscellaneous Small Gram Negative Rods*

All from the patients in Group II These three strains all differed from each other in morphology and other characters They differed from the possible *H vaginalis* group in morphology 2 were beaded and Gram variable One was a thin weakly-staining Gram-negative rod Colonially, they resembled the *H vaginalis* type to a certain extent at 48 hours, but later developed colonial differences The colonies were, in general, smaller than those of the other types Both aerobic and

microaerophilic growth in thioglycollate were represented in this group The strain showing satellitism was not checked for its X and V needs

(d) *3 Gram Negative to Gram-Positive Rods*

All from the patients in Group II Although this phenomenon was common, these strains needed many films to establish their Gram-positivity, and so were included

Several such strains were isolated, but only three were examined in detail At 24 and 48 hours, the colonies of these 3 resembled the *H vaginalis* type, but later became flat and irregular They differed from each other in morphology and it was not suggested that they formed a homogeneous group All these strains showed microaerophilic growth One strain needed CO_2 to grow on McLeod's medium

(e) *Gram-Positive Rods*

4 were examined in detail and 26 for their antibiotic sensitivity only The strain showing satellitism was not checked for its X and V needs All were from the patients in Group II

Antibiotic Sensitivity

All the *H influenzae* type were resistant to bacitracin, and either resistant or moderately sensitive to penicillin All the other types were sensitive to bacitracin and highly sensitive to penicillin, giving much larger zones than the *H influenzae* type The strains in all groups were sensitive to streptomycin, aureomycin, and chloramphenicol, except the atypical strain from a patient in Group II, which was relatively resistant to aureomycin and showed a lesser degree of sensitivity to chloramphenicol and streptomycin than the other strains About half of the strains in each group were resistant to sulphathiazole

In view of the reports of the resistance of strains of *H vaginalis* to various antibiotics, 30 strains of small Gram positive rods were tested and 2/30 showed moderate resistance to penicillin, streptomycin, aureomycin and bacitracin None showed any to chloramphenicol

No strains showed *motility* *spores* were not seen nor did any grow upon nutrient agar *Capsules* were not demonstrated but were not extensively looked for

The poorer growth on blood media of the *H influenzae* type may be due to V factor destroying substances in sera and especially red blood cells of various species (*Waterworth 1951*) *Amies & Jones* noticed *inhibition by sheep blood* *In semi solid* Casman's medium the growth was either confluent extending along the stab or discrete colonies formed sometimes fluffy The strains showed little difference in appearance Presumably these are the puff ball colonies described by *Gardner & Dukes* All the strains grew aerobically Most of the strains of all groups grew anaerobically but this point was not fully investigated There is some doubt whether *H influenzae* will grow anaerobically (*Topley & Wilson* p 904) Bergey's Manual considers it as a facultative anaerobe

The viability was low and all but two days on Casman's medium at daily subculture was more reliable

or two longer In semi solid media they lived about a week The *H influenzae* type lived longer on all media A heavy inoculum was better for all of the more delicate strains

The heat resistance was low for the few strains tested They were killed by 50° C for half an hour This was substantially the same among all the groups

All grew at 30° C within a week on Casman's agar, except 3 of the miscellaneous and Gram negative to Gram positive strains At 22° C, a few of the *H influenzae* type and of the miscellaneous and Gram negative to Gram positive strains grew None of the possible *H vaginalis* group grew

The strains withstood freeze drying all but the last few strains have been revived from the freeze dried state

By the paper technique, *H influenzae* type were oxidase positive The strains positive by this technique were negative or weak positive when the same dye was

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on to the production of acid

influenzae in sugar free media and *Lunevall* (1951) confirmed this, and showed the stimulating action of small amounts of some carbohydrates on this acid production although no carbohydrate was utilized The sugar reactions of *H influenzae* have been variously reported With many of the strains in all groups, there was some acid production in the controls and so fermentation was assumed when there appeared more acid in the test than in the control Other strains gave more clear cut results with a negative control With others, there was fermentation in all of the tubes

In general acid was produced in glucose by almost all the strains, in laevulose and dextrin by most and in lactose mannitol and sucrose by random strains throughout the groups No gas production was observed with any strain Sugar fermentation carried out by this technique did not appear particularly useful in distinguishing the strains

Serology

Of 25 strains *Amies & Jones* using an agglutination test, were able to show two groups and 2 odd strains but they had difficulty from auto agglutinability, and suggested that their strains become auto-agglutinable after continued subculture For the present

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type Further work has

Incidence of the Types in the Patients in Group II

All the strains of *H influenzae* type isolated from patients were derived from the patients in Group I Although these specimens came from both sexes all the strains were isolated from female patients No strain of this type was found in patients in Group II Possibly this is due to the low incidence of *H influenzae* type, 1 5 per cent to 3 per cent in Group I Both Group I and Group II specimens were plated on

strains in Group I came from cervical specimens

The low incidence of *H vaginalis* type strains from the Group I plates

is artificial Experience had not been acquired in the work Many strains died and many we rejected Only 6 were kept from the several hundred plates in order to carry out a preliminary survey of the media and methods

The distribution of the strains, of the presence of small Gram-negative rods on the films and of clue cells, is shown in Table 4

TABLE 4

Incidence of the Strains Isolated in the Investigation

Organisms grown	Strains	116 women aged 11 to 55 years					
		30 cases vaginitis	8 cases vaginitis & cervicitis	20 cases cervicitis	6 cases misc	20 cases discharge	32 normal patients
Gram-negative rods grown	33	11	1	7	1	6	7
a) Possible *H vaginalis*	15	6	1	3	1	2	2
b) Miscellaneous	7	2	0	3	0	1	1
c) Gram negative to Gram positive	11	3	0	1	0	3	4
Small Gram-negative rods in smear		20	6	12	4	10	17
All clue cells		9	1	5	3	8	6

There are more strains from patients in this Table than in Table 3 because some of the strains were lost and also because not all the Gram negative to Gram-positive strains recorded in this Table were examined in detail

The number of cases in this series was low, but a few inferences can be made from Table 4 and other data not in the Table A much larger series of cases is desirable to demonstrate the validity of some of these points

It can be seen that the isolation of small Gram-negative rods is associated irregularly with the clinical condition, although there is a tendency for possible *H vaginalis* strains to be cultured slightly more frequently from cases of vaginitis of cervicitis compared with cases of discharge and normal patients, and more so from the patients with disease than from the normal patients Percentages are not given, as the numbers are considered too low

Organism of all kinds were numerous in 28 5 per cent of the smears, but this finding was not especially associated with any group of patients Small Gram-negative rods were present, in greater or lesser numbers on 59 per cent of the smears, but these cases were not predominately distributed in any clinical condition

Small Gram-negative rods on the film tended, though not uniformly, to be accompanied by the isolation of small Gram-negative rods from the specimen It made no difference whether the rods seen were numerous, moderate or few The specimens in which the smears had small

Gram negative rods (59 per cent) yielded 25 out of 33 of the strains of small Gram negative rod isolated those smears without (41 per cent) yielded 8 The *H vaginalis* type isolations showed the same distribution as other small Gram negative rods

Of the 116 specimens small Gram positive rods were seen in 63 per cent of the smears and cultured in 75 per cent small Gram negative rods in 59 per cent and cultured in 28 5 per cent

In all the smears where there were numerous small Gram positive rods then small Gram negative rods also occurred Similarly where small Gram negative rods were numerous small Gram positive rods occurred Also in a majority of the smears where moderate or few small Gram positive rods occurred then small Gram negative rods were present and vice versa Thus many of the small Gram negative rods seen on the film may have been decolourized cells from Gram positive strains This may be one reason why the small Gram negative rods in the smears sometimes failed to grow on culture In addition the media may have been unsuitable for some strains and antagonism between species may have occurred on crowded plates Also some of the small Gram negative rods seen on the films may have been obligate anaerobes Vibrio like forms and fusiform small rods on the films were not recorded as small Gram negative rods

Clue cells occurred on 32 smears 3 were true clue cells 22 were mixed clue cells and 7 false clue cells None of these types of cells showed a significant association with any clinical condition They were commoner when there were numerous organisms on the film The isolation of *H vaginalis* type strains was commoner when clue cells of any type had been found on the smear 7 were isolated from 28 per cent of the specimens where clue cells occurred and the other 8 from the remaining 72 per cent Miscellaneous and Gram negative to Gram positive strains showed an even distribution with regard to clue cells However only a third of the specimens which had clue cells on them yielded Gram negative rods in culture This casts doubt on their value in diagnosis of the condition

Leucocytes were more common in pathological conditions but showed no significant association with the isolation of small Gram negative rods

DISCUSSION

fe [illegible]

is [illegible] *para influenzae Haemoph* [illegible]

[illegible]

[illegible] Jones description suggests that their strains were of this type and 8 out of 10 of their strains examined

in this work were indistinguishable from *H influenzae* and 1 from *H para influenzae* They suggest a low incidence of *H vaginalis* However, they used serum yeast agar which might not support the growth of the *H vaginalis* type, or if it did, only badly *Deming*, who used blood agar for isolation, considered his strains to be *H influenzae*, but does not state the incidence The significance of *H influenzae* in this site is unknown, especially in view of the frequent occurrence of non-capsulated *H influenzae* in the respiratory tract without disease The infrequence of indole positivity, and the cross reactions in serology shown by *Amies & Jones* suggest that many of these strains may be non-capsulate *H influenzae* It is perhaps surprising that *H influenzae* is not reported more frequently and was not reported from the larger series *e g*, *Gardner & Dukes'* 1,200 cases, *Gardner, Dampeer & Dukes'* 3,000 cases However, the colonies do not resemble the *H vaginalis* type, and possibly it is rarer in general gynaecological practice than in suspect cases of gonorrhoea *Deming* thought that many of his cases were due to the patient's sexual habits, with the primary source of *H influenzae* being the mouth and respiratory tract In routine gonococcal diagnosis, *H influenzae* would not normally be investigated because the colonies do not resemble those of gonococci and the oxidase reaction is negative or very weak if the dye in poured on to the plate

21 of the other fastidious Gram-negative rods resembled the stock strain from *Gardner & Dukes*, and, from their description, Leopold's *strains and Lutz & Wurch's appear to belong to this type, although the* strains of the two latter were β lytic This possible *H vaginalis* type shows similar characters but some differences in morphology However, it is difficult to be certain that the strains can justifiably be classed in a single group, both because an arbitrary decolourization time is used to separate the strains from very similar Gram-positive organisms and, also because it is uncertain whether this group consists of similar members in view of the small number of tests carried out, and the fact that the criterion in many of the tests is negativity

The taxonomic position of *H vaginalis*, if it is a group, is doubtful It is not established that it belongs to the *Haemophilus* genus if need for X and V factors is taken as a criterion of this genus, as these strains certainly have not the need for X and V factors of *H influenzae* However, the X and V needs of *Haemophilus putorium* and *Haemophilus citreus* are not exactly determined, and *Haemophilus piscium* needs di phosphothiamine but not X or V factors for growth, while *H canis* needs only X factor, although this species possibly resembles *Pasteurella* more than *Haemophilus* The strains differ from *Haemophilus spp* in other ways, such as absence of filaments, oxidase reaction failure to grow on Fildes medium Of the *Haemophilus* genus these strains perhaps resemble *H citreus* most, but this organism grows on X and V agar, produces filaments, produces indole and was isolated from cases of exanthem coitale in cattle, (Bergey's Manual) *Bordetella pertussis*

and *Bordetella para pertussis* were removed from *Haemophilus* to *Bordetella*, as their need for blood was largely to neutralize toxic factors, and *H vaginalis* also shows inhibition by toxic factors Two strains of *B pertussis* and one of *B para-pertussis* were examined by the tests described in this paper, and did not show much similarity to *H vaginalis* These strains of *Bordetella* were microscopically different, slower growing, penicillin resistant and showed many other differences A strain of *H ducreyi* was also examined early in the work but did not appear similar to the *H vaginalis* type colonially nor in its nutritional needs The *H vaginalis* strains do not resemble *Brucella* or *Pasteurella* (descriptions—Bergey's Manual, Topley & Wilson's textbook), though strains of these organisms were not examined in parallel They do not appear to resemble *Lactobacillus*, differing in morphology, in oxygen needs, and other characters, but in view of their absence of catalase it would be interesting to test the growth of these strains at various acidities and on typical lactobacillary media such as tomato juice agar It seems also possible that they are a group of Gram negative or variable *Corynebacteria*, in the wider sense, in other words "diphtheroids", or various poorly-staining strains of such organisms not comprising a homogeneous group In many respects the few Gram-positive small rods examined in detail showed similar characters Growth on tellurite, further biochemical tests, cell wall analysis, serology, and examination of the Gram reaction at an early stage of growth, such as 4 hours, might be of value

The rest of the strains examined seemed to be a heterogeneous group, some of which were similar colonially and in other characters to the *H vaginalis* group and were extremely liable to confusion with it They may be Gram-negative, Gram-variable, depending on the stage of the culture and other factors, or frankly Gram-positive However, unless a very exact technique was used they could easily be confused with the *H vaginalis* type

This work shows that there are many pitfalls in the diagnosis of *H vaginalis* vaginitis, if this is a clinical entity and if *H vaginalis* is a species Factors such as diagnosis by methods where culture is not used or is unsuccessful, absence of detailed investigation of the strain, over-decolourization in Gram staining, staining of 48 hour cultures and others may cause confusion They may also explain the high incidence of the organism found in some of the series of cases described Unfortunately, the number of cases investigated in this paper was low The incidences are given in Table 4, and it can be interpreted that the organism is little commoner in vaginitis than in other pathological conditions, but commoner in pathological conditions than in normal women In view of the high cure rates claimed for sulphonamide preparations, it is possibly of interest that about half the strains of all types showed sulphathiazole resistance

Possibly the vaginal flora in America, Canada and Denmark is dif-

ferent *Doll* in Germany, in a short report, stated that he found no *H vaginalis* using the methods of *Gardner & Dukes*

It is also possible that there was an epidemic of some bacterial condition or possibly a viral disease both in Leopold's and Gardner & Dukes' areas *Gardner & Dukes* (1955) successfully infected volunteers However, it was much easier to cause artificial infection with discharge from cases than with pure cultures The use of filtered discharge is not described, so it is not possible to disprove a virus infection

It is difficult to explain the absence of *H vaginalis* type strains in a series of normal women and cases of cervicitis (*Gardner & Dukes* 1955), and of vaginitis (*Doll* 1958) *Lutz & Burger* (1958) found them commonest in trichomonas cases, next in normal pregnancy then in leucorrhoea, and the other authors remark on their frequent association with *T vaginalis* This series was not long enough to investigate this point In the other previous reports, the vaginitis is described as mild in most cases, and, in some cases, as more or less symptomless One is struck by the mildness of the cases, and this is perhaps borne out by the rarity of pus cells *Amies & Jones* found pus cells common *Gardner & Dukes* (1955) and *Lutz et al* (1956) and others, comment on the absence of pus cells, perhaps a rather unusual finding in an acute bacterial inflammatory condition Certainly in this series pus cells were commoner in vaginitis and in cervicitis than in women with "simple' discharge or normal women, but were not specially associated with the culture of small Gram-negative rods

Clue cells in stained films do not appear of much value because epithelial cells with only Gram-negative rods on them are rare, and partly because cells with a mixed flora on them show little association *with the growth of small Gram-negative rods*

It seems possible that small Gram-negative rods occur in the normal vagina, and under pathological conditions find the environment better for growth perhaps a change of pH, suppression of antagonists or such factors occur under these circumstances Some of these strains may be *H influenzae* of *H para-influenzae*, and some may form a homogeneous group which can be called *H vaginalis,* though more work is needed to establish this What part they play in the causation of vaginitis needs a larger series with careful clinical classification and bacteriological examination of the strains isolated

SUMMARY

A series of stock strains of *H vaginalis*, *H influenzae* and *H para-influenzae* and strains isolated both from routine gonococcal diagnosis plates and from specimens from a clinically classified series of 116 patients were compared

They fell into a group of *H influenzae* or *H para influenzae* type strains, a group resembling *H vaginalis* as described by *Gardner &*

Dukes (1955), and a miscellaneous group of organisms The strains classed as *H vaginalis* were shown to differ in some characters, yet the strains resembled one another to a considerable extent The taxonomic position of these strains is uncertain

No strong evidence for a causal relationship to vaginitis could be demonstrated in this short series of patients

Stress is laid on the importance of careful Gram staining and detailed investigation of small fastidious rods found in vaginal specimens before they can be categorized as *H vaginalis*

REFERENCES

[illegible]

... *Halpern B & Thomas B S* Hemophilus vaginalis vaginitis Amer J Obstet Gynec 74 834 1957

Bret A J & Cohen Debray Vaginites a "Hæmophilus vaginalis" Presse Med 67 1611 1959

Casman E P ... infusion blood agar base for Neisseriae pneumococci and streptococci Amer J Clin Path 17 281 1947

Cook C T A plate test for nitrate reduction J Clin Path 3 359 1950

Denning J E Haemophilus influenzae vaginitis North W Med (Seattle) 54 999 1955

Doll W Vorkommen von "Haemophilus vaginalis" bei "unspezifischer Vaginitis"? Zbl Bakt I Abt Orig 171 372 1958

Duguid J P The demonstration of bacterial capsules and slime J Path Bact 63 673 1951

Dukes C D & Gardner H L Studies on the growth requirements of Haemophilus vaginalis Bact Proc p 114 1958

Gardner H L & Dukes C D New etiologic agent in nonspecific bacterial vaginitis Science 120 853 1954

Gardner H L Dampeer T K & Dukes C D The prevalence of vaginitis a study in incidence Amer J Obstet Gynec 73 1080 1957

Gardner H L & Dukes C D Haemophilus vaginalis newly defined specific infection previously classified nonspecific vaginitis Amer J Obstet Gynec 69 962 1955

Henriksen S D [illegible]

[illegible]

Lut ... *Hurch Th & Crotten O* Quelques données sur les "petits bacilles Gram negatif" agents d une leucorrhée individualisée Gynec et Obstet 55 75 1956 a

Lut A Grotten O & Wurch Th Étude des caractères culturaux et biochimiques de bacilles du type Hemophilus hemolyticus vaginalis Leur sensibilite aux antibiotiques Rev Immunol (Paris) *20* 132 1956 b
Lut- A & Burger P Les Infestations à Trichomonas Masson p 175 1957
Mackie T J & McCartney J F Handbook of Practical Bacteriology 9th ed E & S Livingstone London 1953
McLeod J Coates J Happold F Priestley D & Wheatley B Cultivation of the gonococcus as a method in the diagnosis of gonorrhaea with special reference to the oxydase reaction and the value of air reinforced in its carbon dioxide content J Path Bact *39* 221 1934
Ray J L & Maughan S M Hemophilus vaginalis as etiological agent in vaginitis West J Surg *64* 581 1956
Reymann F Cultivation of gonococci as diagnostic method in gonorrhoa in women I Morphological and cultural aspects of gonococci Acta derm venerol *24* 130 1943
Rosher A B Hæmophilus influenzae and its relation to epidemic influenza Proc Roy Soc Med *40* 749 1947
Stuart R D Toshach S R & Patsula T M The problem of transport of specimens for culture of gonococci Canad J Publ Hlth *45* 73 1954
Thompson L Other oxydase positive bacteria found in cultures made for Neisseria gonorrhoeae J Bact *31* 82 1936
Topley & Wilson Principles of Bacteriology & Immunity 4th ed revised G S Wilson & A A Miles 1955
Tunnicliff G Studies on Haemophilus influenzae biochemical activities Acta path et microbi l scandinav *29* 387 1951
Waterworth P M The stimulation and inhibition of the growth of Haemophilus influenzae on media containing blood Brit J Exp Path *36* 186 1955
Wurch Th & Lut- A Étude du contenu vaginal dans 500 cas de leucorrhees Cytologie Microbiologie Revue francaise Gyn Obstet *50* 289 1955

DEVELOPMENT OF THE LEWIS BLOOD GROUP IN THE NEWBORN

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The so called Lewis blood group was originally discovered by *Mourant* (1) who based upon studies of a few families considered the new factor to have a dominant inheritance not excluding a recessive heredity.

Andresen (2) independently discovered an antibody to the same bloodtype and named the factor Le. Andresen tested mothers and children with his antiserum and found a distribution of the new bloodtype indicating that mothers who were Le positive from a genetical point of view had to be considered homozygotes, while infants which were Le positive might be homozygotes as well as heterozygotes for the Le gene. Andresen found a distribution as follows:

Mothers			Children							
			0–3 months		4–6 months		9 months		10–12 months	
Type	No.	%	No.	%	No.	%	No.	%	No.	%
Le+	166	21	78	79	74	73	18	36	19	29
Le−	618	79	21	21	28	27	32	64	46	71

From the table it will be seen that the Le gene becomes demonstrable also in single dose in the cells of the infants during the period from birth to the age of six months. Andresen verified his theory by further studies of complete families.

The simple recessive inheritance of the Lewis factor was also verified by *Race et al.* (3) in a study of 79 families. Further evidence to support the recessive inheritance of the Lewis factor was revealed by the studies of *Grubb* (4) who showed that adult Lewis positive individuals were non secretors in the ABO system, i. e. homozygotes for the non secretor gene. Similarly it was found that Lewis positive individuals secreted Lewis substance in their saliva (4, 5).

The next phase in the development of this system was *Andresen*'s (6) finding of an antibody which agglutinated the cells of most Lewis

This work was carried out in 19 3.

negative individuals This new factor which Andresen called L_2 was fully developed in O and A_2 cells but often less marked in A_1 cells

The nomenclature for this system was changed in 1949 (7) The gene for the original Lewis (L_1) type was called Le^a and that for the L_2 type Le^b

Individuals of type Le (a+b—) are genetically	Le^a Le^a
Individuals of type Le (a—b+) are genetically	Le^b Le^b or
	Le^a Le^b or
	$Le^?$ Le^b

$Le^?$ indicates additional allelomorphs in the theory

The incidence of these blood groups in this country are (8)

Le(a+b—) 18 per cent, Le(a—b+) 75 55 per cent, Le(a—b—) 6 45 per cent

These figures originate from a study of 1055 blood donors tested with anti-Le^a sera and an anti-Le^b serum which agglutinated A_1 cells at a similar scale as O and A_2 cells

The purpose of the present study has been to investigate the development of the phenotype Le(a+b—) in newborn and to determine whether an actual difference exists as to the development of this factor between matures, prematures and infants with erythroblastosis A similar study indicated that the Lewis group is but sparingly present in the blood before birth in infants which are homozygotes for the Le^a gene (9) *Rosenfield & Ohno* (10) found that cord blood gave the reaction Le(a—b—) *Unger* (11) tested 102 cord bloods and all were Le(a—)

METHODS

The bloods were tested against two anti Le^a serums and one anti Le^b serum A four per cent suspension of the cells in isotonic saline was made Two volumes of the test serum and one volume of the cell suspension were incubated for two hours at room temperature in Rh tubes Three grades of agglutinability with anti Le^a serums were distinguished as follows

— no agglutination
(+) *faint agglutination*
\+ *definite agglutination*

RESULTS

Phenotype Le(b+) could not be demonstrated in any of the samples tested *The development of the phenotype Le(a+) was found to be* independent of the ABO group of the cells These data have not been listed in the table

According to the table is the phenotype Le(a+) faintly present in the cells of some infants even in the first week of life In the total number of observations during the first week of life, 10 of 45 samples or 22 per cent were weakly Le(a+) As early as in the second week of life a radical changes has taken place as indicated by the fact that the cells now in the majority of cases are either weakly or definitely Lewis

positive The change in agglutinability shows no marked difference in the four groups into which the material is divided The agglutinability appears more rapidly than what can be ascribed to the postnatal production of erythrocytes Consequently it may be concluded that the Lewis receptor is actually present but hardly demonstrable in the erythrocytes produced before birth After birth, however, the cells will be "changed" within a couple of weeks whereby the Lewis receptor becomes easily demonstrable

TABLE 1
Age of the Children in Days

		0 7	8 14	15 21	22 28	29 35	36 42	43 49	50 56	57 63	64
Children born to normal time	a —	11	1	1	–	2	–	–	1		1
	a(+)	3	3	1	1	1	1	6	1	2	4
	a +	–	2	4	2	2	2	1	2	3	2
Premature until 6 weeks to early born children	a —	8					–				
	a(+)	4	1	1					–		
	a +		2	4	4	1	–				–
Premature 6 weeks or more to early born children	a —	12	2			1			–		–
	a(+)	1		3	1	1	3	1	1		–
	a +		2	1	6	5	2				
Children suffering from erythroblastosis	a —	4						–			–
	a(+)	2		1							–
	a +						–		–		–
Total distribution	a —	35	1	1		3			1		1
	a(+)	10	4	6	2	2	4	7	2	2	4
	a +		6	9	12	8	4	1	2	3	2
Number of children		45	13	16	14	13	8	8	5	5	7

TABLE 2
Total Distribution

In first week of life		After 15th day of life	
a —	35	a —	6
a(+)	10	a(+)	29
a +		a +	41

REFERENCES

1. *Mourant A E* A "new" human blood group antigen of frequent occurrence Nature *158* 237 1946
2. *Andresen P H* Blood group with characteristic phenotypical aspects Acta path et microbiol scandinav 25 616-618 1948
3. *Race R R Sanger Ruth Lawler Sylvia D & Bertinshaw Doreen* The Lewis blood groups of 79 families Brit J exp Path *30* 73 83 1949
4. *Grubb R* Correlation between Lewis blood group and secretor character in man Nature *162* 933 1948
5. *Brendemoen O J* Studies of agglutination and inhibition in two Lewis antibodies J Lab clin Med 34 538 542 1949

6 *Andresen P H* The blood group system L A new blood group L A case of epistasy within the blood groups Acta path et microbiol scandinav 25 728 731 1948
7 *Andresen P H Callender Sheila T Fisher R A Grubb R Morgan W T J Mourant A E Pickles Margaret M & Race R R* A notation for the Lewis and Lutheran blood group systems Nature *163* 580 1949
8 Unpublished observations
9 *Brendemoen O J* Nyoppdagede blodtyper (Lewis og S) Nordisk Medicin *40* 1789 1948
10 *Rosenfield R F & Ohno G* Unpublished observations cited by Race R R & Sanger Ruth in Blood Groups In Man third edition
11 *Unger L J* Unpublished observations cited by Race R R & Sanger Ruth in Blood Groups In Man third edition Blackwell Scientific Publications Oxford 1958

DEPARTMENT OF BACTERIOLOGY UNIVERSITY OF GOTHENBURG

STUDIES ON THE ANTIGENIC FACTORS IN A GROUP A STREPTOCOCCAL CULTURE FILTRATE

By

L A HANSON and S E HOLM

Received 19 xi 60

In our earlier studies on streptococcal antibodies in human sera we have found numerous antibodies of different specificity corresponding to various streptococcal antigens (6 7 10) With very few exceptions several of these antibodies were demonstrated in sera from adults whereas in sera from children the number of antibodies was low but gradually increased in relation to age to the adult level To get a better understanding of these findings and their relation to streptococcal diseases we found it necessary to try to characterize the antigenic factors formed by the β hemolytic streptococci Several authors have shown that these bacteria give rise to a large number of antigenic substances during the course of their growth (1 3 4 5 6 7 9 11 14 19 and others) In the present investigation we have tried to separate and purify these substances by salt precipitation and ion exchange chromatography

MATERIAL AND METHODS

Antigens Filtrates were made of 18 hour cultures of β hemolytic streptococci (S84 Lancefield group A Griffith type 3) grown in beef he [illegible]

[illegible]

[illegible] An immune serum fr m a h rse immunized with stra n Dochez NY 5

[illegible] O Ouchterlony is thanked for current interest and advice and Assoc Prof S P Halbert New York f r the most valuable substances he made available to us Drs J Coa and B Johansson Goteborg are thanked for ad[illegible] ce and instruction c n cerning [illegible] H rsch [illegible] istance of Mmes [illegible] support [illegible] The investigation was

STREPTOCOCCUS HAEMOLYTICUS STRAIN S 84

GROUP A (LANCEFIELD)

TYPE 3 (GRIFFITH)

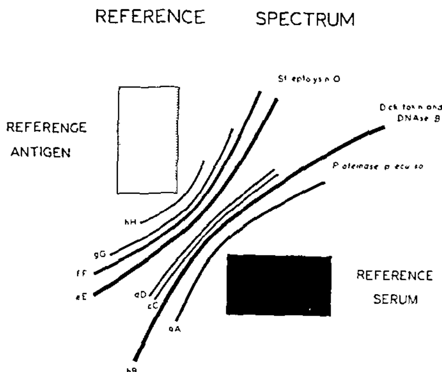


Fig. 1
Diagrammatic representation of the reference spectrum

(Lancefield group A, Griffith type 10) was used for the immune electrophoretic analyses.

Serum from a patient with a long history of mitral stenosis on a rheumatic fever base (20, Case No. 4) was used as a reference serum for the double diffusion analyses because of its high antibody content (6).

Reference spectrum. Throughout the investigation all of the double diffusion analyses were performed as comparative analyses of the tested substance and the reference antigen by means of the reference serum. The reference spectrum was formed by *the ten times concentrated culture filtrate of the strain S84 and the* human reference serum. The precipation pattern obtained consisted of eight serologically separate precipitating systems labelled *aA* to *hH* (Fig. 1).

Purification of antigen. Salt precipitation of the antigenic factors was performed by means of precipitation of the filtrates with Na_2SO_4 at full saturation and with $(NH_4)_2SO_4$ at 20, 40, 60, 80 and 100 per cent saturation at room temperature (20°). The preparations were tested for protein with Markham's method for micro-Kjeldahl analysis (13).

Separation of the antigenic factors in the filtrates was obtained by anion as well as cation exchange chromatography according to *Peterson & Sober* (16). The anion exchange chromatography experiments were performed on DEAE cellulose (Eastman & Kodak). It could not be used without through alternate washings with *M* NaOH and *M* HCl. Columns 2.4 cm in diameter were filled with the exchanger suspended in the initial buffer. After settling the substance was packed to a constant height of about 6 cm and *200–300 mg of protein was added to the column. For the* elution phosphate buffers *pH* 6.8 were used with stepwise increases in ionic strength. Fraction I was eluted with 0.001 *M* phosphate buffer, fraction II with 0.005 *M* phosphate buffer + 0.005 *M* NaCl and fraction III with 0.02 *M* buffer + 0.02 *M*

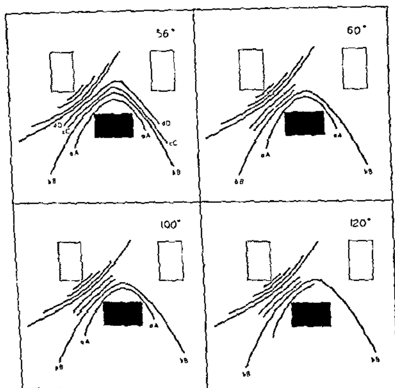


Fig 2
Diagrammatic representation of comparative analyses of the reference spectrum and the heat treated antigen

NaCl Finally fraction IV was eluted with 0.1 M buffer + 0.1 M NaCl The effluent was [illegible] ncentration in each fraction [illegible]

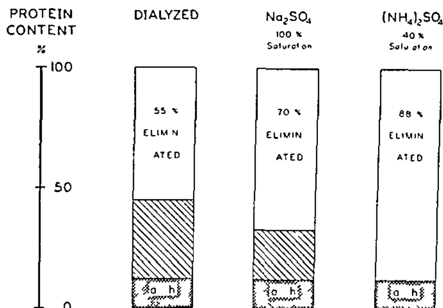


Fig 3
Diagram of the purification of the reference antigen by salt precipitation and dialysis

Diffusion in gel technique The antigenic factors of the different fractions were analyzed by diffusion in-gel methods The double diffusion method of *Ouchterlony* (15) was used as described by *Hanson* (6) The immune electrophoresis of *Grabar* & *Williams* (2) was applied as modified by *Wadsworth* & *Hanson* (18) as well as the comparative immune electrophoresis described by the latter authors (18) The electrophoretic separations were performed in a sodium veronal buffer of pH 8.4 with a potential gradient of 5V/cm in the agar for 70 minutes The localization of the hemolytic activity of streptolysin O after electrophoresis was determined using the technique described by *Hanson Raunio* & *Wadsworth* (8) The activity was seen as hemolysis of sheep blood cells put in basins made in a plexiglass matrix which was placed on top of the agar after the electro separation

RESULTS

Identification experiments with known antigens By means of the isolated streptococcal antigens some of the antigenic factors in the reference culture filtrate could be identified It was then found that factor *a* corresponded to proteinase precursor, whereas factor *b* identified with both the Dick toxin preparation and the streptococcal DNAse B Comparative analyses of these two antigenic preparations showed a reaction of non-identity Factor *e* identified with streptolysin O The preparations of DPNase and streptokinase could not be referred to any of the factors *a-h*, but streptokinase gave a precipitate with the refer-

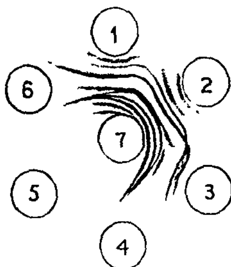


Fig 4
Photo of a drawing of a double diffusion analysis showing the antigenic factors in the supernatants obtained by salt precipitation of the reference antigen with $(NH_4)_2SO_4$ at different concentrations 1 reference antigen 2 20 per cent saturation 3 40 per cent 5 80 per cent 6 100 per cent saturation of $(NH_4)_2SO_4$ and 7 reference serum

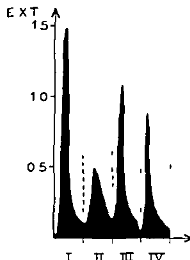
ence serum In the same way some of the lines could be identified in the spectrum obtained with the reference antigen and the horse immune serum (7)

Characterization experiments on the antigenic factors Some of the antigenic factors differed in heat sensitivity Thus the factors *e, f, g* and *h* were destroyed after 60 min at 56°, the factors *c* and *d* at 100° C Heating to 120° for the same length of time left only factor *b* (Fig 2)

In order to purify the antigenic factors experiments were performed to eliminate broth proteins, non antigenic substances and antigens not reacting with the used immune serum (Fig 3) Dialysis of the filtrates eliminated 55 per cent of the protein content without loss of any of the antigenic factors *a h* Na_2SO_4 at 100 per cent saturation did not precipitate any of the antigenic factors *a h*, but did precipitate 30 per cent of the protein material in the dialyzed culture filtrates Precipitation with $(NH_4)_2SO_4$ at 40 per cent saturation and dialysis of the supernatant reduced the protein content by 88 per cent without affecting the factors *a h* At a saturation higher than 60 per cent these factors were all found in the precipitate This is illustrated by the double diffusion analysis shown in Fig 4

Attempts to separate the demonstrated antigenic factors were made by means of anion and cation exchange chromatography The separa-

SEPARATION
OF REFERENCE ANTIGEN
BY ANION EXCHANGE CHROMATOGRAPHY AT pH 6.8



	ANTIGENIC a	b	c	d	FACTORS e	f	g	h	STREPTO LYSIN O
REFERENCE ANTIGEN	+	+	+	+	+	+	+	+	+
FRACTION I	+	(+)	++						+
II	(+)	+							−
III	(+)	++	+	+	+				+
IV		(+)	(+)	(+)					+

Fig. 5

Results of the fractionation on DEAE cellulose after salt precipitation of the reference antigen with $(NH_4)_2SO_4$ at 40 per cent saturation. The elution was made with phosphate buffers pH 6.8 of stepwise increasing ionic strengths and with an addition of NaCl. The table shows the antigenic factors and the streptolysin O activity in the different fractions.

tions were performed on the culture filtrates after precipitation with $(NH_4)_2SO_4$ at 40 per cent saturation. A typical fractionation on DEAE-cellulose is shown in Fig. 5. The comparative double diffusion analysis of the fractions I–IV with the reference spectrum showed that some separation of the antigenic factors could be obtained (Figs. 5 and 6). Fraction I showed a concentration of factors *a* and *c* while factor *b* was present in a low amount. The second fraction contained factors *a* and *b*. The third fraction showed the presence of factors *a*, *b*, *c*, *d* and *e* with an increased concentration of factor *b* compared to the reference antigen. Finally, in fraction IV factors *b*, *c* and *d* were found in low amounts. Streptolysin O activity was found in fractions I, III and IV.





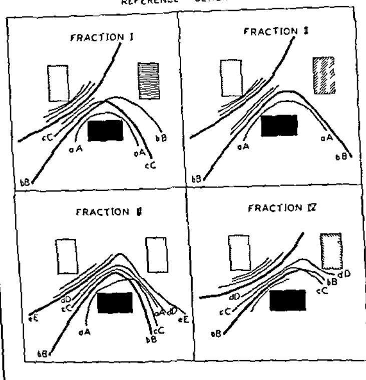
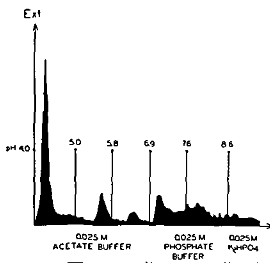


Fig 6
Double diffusion analyses of the fractions obtained by separation on DEAE cellulose of the reference antigen

A typical curve from a fractionation on CM cellulose is seen in Fig 7 These columns were eluted with buffers of increasing *p*H On equilibrating the antigenic preparation with the starting buffer at a *p*H lower than 4.0 a precipitate was noted This precipitate was dissolved and called fraction I It was found to contain all the factors *a*–*h* (Fig 8) Streptolysin O activity could also be demonstrated Factor *b* could be isolated at *p*H 5.8 (fraction IV in Fig 7) Fraction V eluted at *p*H 6.9 showed the presence of the antigenic factors *a* and *b* The factor *b* was present in a very low quantity however as it was only indicated by a

SEPARATION OF REFERENCE ANTIGEN

BY CATION EXCHANGE CHROMATOGRAPHY



	ANTIGENIC				FACTORS				STREPTOLYSIN
	a	b	c	d	e	f	g	h	O
REFERENCE ANTIGEN pH 76	+	+	+	+	+	+	+	+	+
FRACTIONS I pH <40	(+)	(+)	+	+	+	+	+	+	+
II-III pH 40-50									−
IV pH 58		++							+
V pH 69	++	(+)							+
VI VII pH >69									−

RECHROMATOGRAPHY OF FRACTION V

pH 69	+	−

Fig 7

Results of the fractionation on CM cellulose after salt precipitation of the reference antigen with $(NH_4)\,SO_4$ at 40 per cent saturation The elution was made with various buffer solutions of increasing *pH* as indicated in the fig The table shows the antigenic factors and the streptolysin O activity in the different fractions Fraction I was obtained as a precipitate when the pH of the antigen sample was adjusted to $< 4\,0$

deviation phenomenon in the comparative double diffusion analysis (Fig 8) Rechromatography of this fraction showed that it was possible to obtain factor *a* in an immunologically pure form A weak streptolysin O activity could be found in fractions IV and V

SEPARATION OF REFERENCE ANTIGEN

BY CATION EXCHANGE CHROMATOGRAPHY

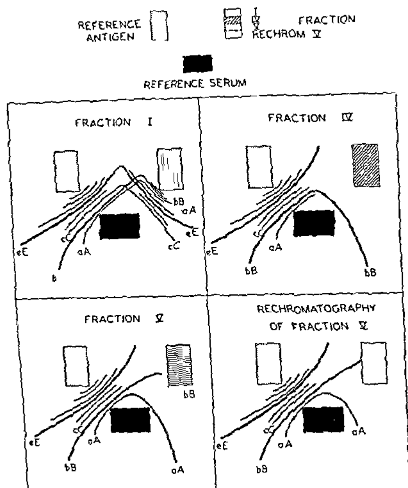
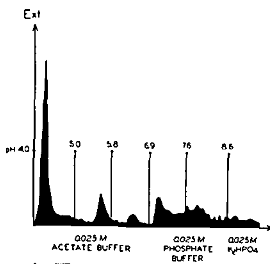


Fig 8
Double diffusion analyses of the fractions obtained by separation experiments on CM-cellulose of the *reference antigen*

Some characteristics of the antigenic factors were also noted in immune electrophoretic analyses These experiments were performed with the *culture filtrate* of the strain C 203 S and the horse immune serum A spectrum of eleven *precipitation lines* was formed (Fig 9 a) By means of the *preparations* of *purified* streptococcal antigens several of these precipitates could be identified using the comparative ...

SEPARATION OF REFERENCE ANTIGEN
BY CATION EXCHANGE CHROMATOGRAPHY



	ANTIGENIC			FACTORS					STREPTOLYSIN
	a	b	c	d	e	f	g	h	O
REFERENCE ANTIGEN pH 7.6	+	+	+	+	+	+	+	+	+
FRACTIONS I pH <4.0	(+)	(+)	+	+	+	+	+	+	+
II-III pH 4.0-5.0									−
IV pH 5.8		++							+
V pH 6.9	++	(+)							+
VI-VII pH >6.9									−

RECHROMATOGRAPHY OF FRACTION V

pH 6.9	+	−

Fig. 7

Results of the fractionation on CM cellulose after salt precipitation of the reference antigen with $(NH_4)_2SO_4$ at 40 per cent saturation. The elution was made with various buffer solutions of increasing *pH* as indicated in the fig. The table shows the antigenic factors and the streptolysin O activity in the different fractions. Fraction I was obtained as a precipitate when the pH of the antigen sample was adjusted to < 4.0

deviation phenomenon in the comparative double diffusion analysis (Fig. 8). Rechromatography of this fraction showed that it was possible to obtain factor *a* in an immunologically pure form. A weak streptolysin O activity could be found in fractions IV and V.

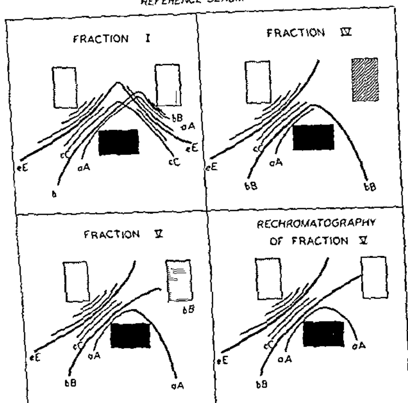


Fig. 8

Double diffusion analyses of the fractions obtained by separation experiments on CM-cellulose of the reference antigen

Some characteristics of the antigenic factors were also noted in immune electrophoretic analyses. These experiments were performed with the culture filtrate of the strain C 203 S and the horse immune serum. A spectrum of eleven precipitation lines was formed (Fig. 9 a). By means of the preparations of purified streptococcal antigens several of these precipitates could be identified using the comparative immune electrophoretic method (Fig. 9 b). Thus it was found that the proteinase precursor had the lowest electrophoretic mobility whereas the two pre-

STREPTOCOCCAL ANTIGEN

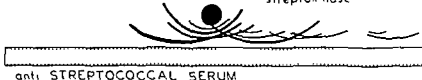


Fig 9 a
Immune electrophoresis analysis of a streptococcal culture filtrate (C 203 S) using a horse immune serum The identified precipitates are designated

STREPTOCOCCAL ANTIGEN



Fig 9 b
Comparative immune electrophoretic analysis showing the identification of the proteinase precursor precipitate The proteinase precursor preparation is compared with the electroseparated streptococcal antigen by means of a horse immune serum

cipitates that corresponded to two antigenic substances in the broth showed the highest mobilities In the region between these precipitates were found precipitation lines corresponding to streptokinase, streptolysin O, streptococcal DNAse B and Dick toxin (Fig 9 a) The localization of the streptolysin O precipitate was in accord with the distribution of the hemolytic activity as determined after electrophoretic separation of the reference antigen using the technique of *Hanson et al* (8)

DISCUSSION

Using ion exchange chromatography it was found that some separation and purification of the antigenic factors could be obtained In these experiments the CM-cellulose was found to be a useful exchanger With this substance as adsorbant it was possible to get two of the antigenic factors, namely, the *a* and the *b* factors, in an immunologically pure form The chromatography of the streptococcal antigens on DEAE-cellulose also showed some separation of the antigenic factors Changing the experimental conditions might possibly give a better separation even if a comparatively good separation was obtained with the rather large elution steps used in the experiments on the DEAE-cellulose It should be noted, however, that the immunological technique used as a

test of the purity of the fractions is a sensitive one and that the presence of very low quantities of antigenic factors could be revealed On the other hand it does not exclude the presence of non antigenic substances or antigens for which the corresponding antibodies were not present in the used immune serum

It was valuable to have the opportunity to include in the investigation the streptococcal antigens isolated and designated by *Halbert* (5) The purity of these substances was confirmed in our experiments and this illustrates the usefulness of the continuous flow electrophoresis and calcium phosphate chromatography used by Halbert for the isolation of these substances

It should be mentioned that the antigenic factor which Halbert first supposed to correspond to erythrogenic toxin he later designated tentatively as DNAse B (5) In our experiments we found that the factor *b* identified with both a preparation of erythrogenic toxin and the DNAse B Comparative analysis of these two antigens showed however reactions of non identity immune electrophoretic analyses showed different mobilities for the two factors and in the spectrum obtained with the horse immune serum they formed two serologically separate precipitates (7) These results indicated that the two precipitation lines corresponding to Dick toxin and DNAse B were superimposed and in the actual reference spectrum were seen as line *bB*

It may seem confusing that streptolysin O activity was found in some chromatographic fractions where the antigenic factor *e* identified as streptolysin O could not be found In control experiments it was noted however that the presence of streptolysin O could be demonstrated in lower quantities by means of its hemolytic activity than by the double diffusion in gel method

This investigation offers the possibility of further evaluation of findings reported earlier on streptococcal antibodies in human sera (6 7) Thus the most common precipitating streptococcal antibody in human sera is anti streptolysin O which is found in almost all sera from adults This may indicate that the antistreptolysin O titration may be a good test for detection of infections with β hemolytic streptococci pathogenic for man Antibodies against DNAse B were found in about half of the sera from adults whereas antibodies against Dick toxin were found in only a few cases Unfortunately streptokinase did not identify with any factor in the reference antigen The reference serum however contained antibodies against this antigen though the donor of this serum had not been treated with any streptokinase preparation Thus antibodies may exist against streptokinase in human sera and this may perhaps lower the therapeutic effect of streptokinase preparations The appearance of antibodies against streptokinase during therapy using this substance is often a problem for the clinician This trouble might be eliminated if a streptokinase could be prepared with retained activity but without antigenicity

SUMMARY

The antigenic factors in a streptococcal culture filtrate (strain S84) were purified and partly isolated by means of salt precipitation and ion exchange chromatography experiments Some of the antigenic factors have been identified with known streptococcal antigens using diffusion-in gel methods Some immune electrophoretic characteristics of these factors are reported The significance of the findings as correlated to the corresponding antibodies found in human sera is discussed

REFERENCES

1 *Bonilla Soto O & Pomales Lebrón A* Variation with age of antistreptococcal antibodies and their correlation with antistreptolysin O titers in human sera Proc Soc exp Biol N Y *102* 337–340 1959
2 *Grabar, P & Williams C A* Méthode immuno electrophoretique d analyse de melanges de substances antigeniques Biochim biophys Acta *17* 67 74 1955
3 *Halbert, S P Swick L & Sonn C* The use of precipitin analysis in agar for the study of human streptococcal infections I Oudin technique J exp Med *101* 539 556 1955
4 *Halbert S P Swick, L & Sonn C* The use of precipitin analysis in agar for the study of human streptococcal infections II Ouchterlony and Oakley technics J exp Med *101* 557–576 1955
5 *Halbert S P* The use of precipitin analysis in agar for the study of human streptococcal infections III The purification of some of the antigens detected by these methods J exp Med *108* 385 410 1958
6 *Hanson L A* Immunological analysis of streptococcal antigens and human sera by means of diffusion in gel methods Int Arch Allergy *14* 279 291 1959
7 *Hanson L A & Holm S* Precipitating antibodies in human sera from different age groups and in colostrum as determined by streptococcal antigens with diffusion in gel methods Acta Pædiat (Uppsala) *50* 7 16 1961
8 *Hanson L A Raunio V & Wadsworth C* A method of determining electrophoretic mobilities of antibodies Experientia *16* 327 329 1960
9 *Harris T N Harris S & Ogburn C A* Gel precipitation of streptococcal culture supernates with sera of patients with rheumatic fever and streptococcal infection Proc Soc exp Biol N Y *90* 39 45 1955
10 *Holm S & Hanson L A* The presence of precipitating antibodies against β hemolytic streptococci in sera from different age groups fetal newborn and child Acta path et microbiol scandinav *51* Suppl *144* 273 276 1961
11 *Jennings R K* Antigens in scarlatinal erythrogenic toxin demonstrable by the Oudin technic J Immunol *70* 181 186 1953
12 *Lowry O H Rosebrough N J Farr A L & Randall R J* Protein measurement with the Folin phenol reagent J biol Chem *193* 265 275 1951
13 *Markham R* A steam distillation apparatus suitable for micro Kjeldahl analysis Biochem J *36* 790 791 1942
14 *Ouchterlony Ö* Antigen antibody reactions in gels Acta path et microbiol scandinav *26* 507 515 1949
15 *Ouchterlony Ö* Diffusion in gel methods for immunological analysis Progr Allergy 5 1 78 1958
16 *Peterson E A & Sober H A* Chromatography of proteins I Cellulose ion exchange adsorbents J amer chem Soc 78 751 755 1956
17 *Prytz B & Jablon J M* Studies on the type specific M fraction of the hemolytic streptococcus Group A J Bact *69* 529 535 1955
18 *Wadsworth C & Hanson L A* Comparative analysis of immune electrophoretic precipitates employing a modified immune electrophoretic technique Int Arch Allergy *17* 165 177 1960
19 *Watson D W* Host parasite factors in group A streptococcal infections Pyrogenic and other effects of immunologic distinct exotoxins related to scarlet fever toxins J exp Med *111* 255 283 1960
20 *Werkö L Eliasch H Bjorlund F & Crafoord C* Circulatory studies in mitral stenosis before and after commissurotomy Ann Surg *135* 290 298 1952

STUDIES ON THE INACTIVATION OF BACTERIAL VIRUSES BY NORMAL HUMAN SERUM

2. *Effect of phage concentration, temperature, pH and molarity on the kinetics of the coli T 2 phage inactivation*[1]

By

LARS OLOF KALLINGS*

Received 5.xi.60

In a preceding paper (*Kallings* 1961) the inactivation of bacteriophage T 2 by normal serum was found to proceed as a zero order reaction. The purpose of the present paper[3] is to study the ratio between the phage and inactivating serum principle, and the influence of temperature, pH and salt concentration on the reaction velocity.

Material and methods

As described in a preceding paper (*Kallings* 1961).

EXPERIMENTAL

Inactivation of fresh phage added to an incubated mixture of phage and normal serum. To investigate the excess of inactivating serum principle in the reaction mixtures ordinarily employed, the following experiment was set up. Three serum dilutions were tested. For each dilution four rows of tubes containing phage—serum mixture were placed in a waterbath at 37° C. At the time intervals listed in Table 1, two tubes of each dilution were chilled, diluted and plated according to the standard procedure. Fresh phage was added to the remaining tubes after two hours. From then on the decrease in phage activity in these tubes was followed at the same time intervals as with the original reaction mixtures. Within the range of serum dilution tested (1:5–1:40), the fresh phages were inactivated at about the same rate as the particles initially present.

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[1] Aided by grants [illegible]

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SUMMARY

The antigenic factors in a streptococcal culture filtrate (strain S84) were purified and partly isolated by means of salt precipitation and ion exchange chromatography experiments. Some of the antigenic factors have been identified with known streptococcal antigens using diffusion-in-gel methods. Some immune electrophoretic characteristics of these factors are reported. The significance of the findings as correlated to the corresponding antibodies found in human sera is discussed.

REFERENCES

1. *Bonilla Soto O & Pomales Lebrón A*: Variation with age of antistreptococcal antibodies and their correlation with antistreptolysin O titers in human sera. Proc Soc exp Biol N Y *102* 337 340, 1959
2. *Grabar P & Williams C A*: Methode immuno électrophoretique d'analyse de melanges de substances antigéniques. Biochim biophys Acta *17* 67 74 1955
3. *Halbert S P, Swick L & Sonn C*: The use of precipitin analysis in agar for the study of human streptococcal infections. I Oudin technique. J exp Med *101* 539 556 1955
4. *Halbert S P, Swick L & Sonn C*: The use of precipitin analysis in agar for the study of human streptococcal infections. II Ouchterlony and Oakley technics. J exp Med *101* 557–576 1955
5. *Halbert S P*: The use of precipitin analysis in agar for the study of human streptococcal infections. III The purification of some of the antigens detected by these methods. J exp Med *108* 385–410 1958
6. *Hanson L A*: Immunological analysis of streptococcal antigens and human sera by means of diffusion in gel methods. Int Arch Allergy *14* 279 291 1959
7. *Hanson L A & Holm S*: Precipitating antibodies in human sera from different age groups and in colostrum as determined by streptococcal antigens with diffusion in gel methods. Acta Pediat (Uppsala) *50* 7 16 1961
8. *Hanson L A, Raunio V & Wadsworth C*: A method of determining electrophoretic mobilities of antibodies. Experientia *16* 327 329 1960
9. *Harris T N, Harris S & Ogburn C A*: Gel precipitation of streptococcal culture supernates with sera of patients with rheumatic fever and streptococcal infection. Proc Soc exp Biol N Y *90* 39 45 1955
10. *Holm S & Hanson L A*: The presence of precipitating antibodies against β hemolytic streptococci in sera from different age groups: fetal, newborn and child. Acta path et microbiol scandinav *51* Suppl *144* 273 276 1961
11. *Jennings R K*: Antigens in scarlatinal erythrogenic toxin demonstrable by the Oudin technic. J Immunol *70* 181 186 1953
12. *Lowry O H, Rosebrough N J, Farr A L & Randall R J*: Protein measurement with the Folin phenol reagent. J biol Chem *193* 265 275 1951
13. *Markham R*: A steam distillation apparatus suitable for micro Kjeldahl analysis. Biochem J *36* 790 791 1942
14. *Ouchterlony Ö*: Antigen antibody reactions in gels. Acta path et microbiol scandinav *26* 507 515 1949
15. *Ouchterlony Ö*: Diffusion in gel methods for immunological analysis. Progr Allergy *5* 1 78 1958
16. *Peterson E A & Sober H A*: Chromatography of proteins. I Cellulose ion exchange adsorbents. J amer chem Soc *78* 751 755 1956
17. *Prytz B & Jablon J M*: Studies on the type specific M fraction of the hemolytic streptococcus Group A. J Bact *69* 529 535 1955
18. *Wadsworth C & Hanson L A*: Comparative analysis of immune electrophoretic precipitates employing a modified immune electrophoretic technique. Int Arch Allergy *17* 165 177 1960
19. *Watson D W*: Host parasite factors in group A streptococcal infections. Pyrogenic and other effects of immunologic distinct exotoxins related to scarlet fever toxins. J exp Med *111* 255 283 1960
20. *Werko L, Eliasch H, Berglund E & Crafoord C*: Circulatory studies in mitral stenosis before and after commissurotomy. Ann Surg *135* 290 296 1952

STUDIES ON THE INACTIVATION OF BACTERIAL VIRUSES BY NORMAL HUMAN SERUM

2 *Effect of phage concentration, temperature, pH and molarity on the kinetics of the coli T 2 phage inactivation*[1]

By

LARS OLOF KALLINGS

Received 5 xi 60

In a preceding paper (*Kallings* 1961) the inactivation of bacteriophage T 2 by normal serum was found to proceed as a zero order reaction

The purpose of the present paper[3] is to study the ratio between the phage and inactivating serum principle, and the influence of temperature, pH and salt concentration on the reaction velocity

Material and methods

As described in a preceding paper (*Kallings* 1961)

EXPERIMENTAL

Inactivation of fresh phage added to an incubated mixture of phage and normal serum To investigate the excess of inactivating serum principle in the reaction mixtures ordinarily employed, the following experiment was set up Three serum dilutions were tested For each dilution four rows of tubes containing phage—serum mixture were placed in a waterbath at 37° C At the time intervals listed in Table 1, two tubes of each dilution were chilled, diluted and plated according to the standard procedure Fresh phage was added to the remaining tubes after two hours From then on, the decrease in phage activity in these tubes was followed at the same time intervals as with the original reaction mixtures Within the range of serum dilution tested (1 5 1 40), the fresh phages were inactivated at about the same rate as the particles initially present

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[1] Aided by grants from the *Swedish Medical Research Council*

[2] The skilful technical assistance of Mrs *Maud Lindberg* and Miss *Birgitta Sundrisson* is gratefully acknowledged

[3] A preliminary report was read at the 1959 Scandinavian Congress of Pathology and Microbiology published in Acta path et microbiol scandinav Suppl *144* 207, 1951

TABLE 1

Time Survival Relation of Fresh Phage Added to Incubated Reaction Mixtures of Phage and Normal Serum

Reaction time min	Per cent survivors					
	Final serum dilution					
	1 5		1 25		1 40	
	A	B	A	B	A	B
10	72	97*				
20	45	52				
30	34	30	112	112	100	96
40	19	20				
60	17	16	68	61	87	87
90	4	6	38	39	61	65
120	3	6	28	23	52	62
150			9	11	37	34
180			8	9	29	28

* Figure deriving from a single experiment, the other data represent the means of two identical experiments

A *Original reaction mixture Total volume 1 ml, 1 × 10⁴ T2 particles, phage and serum (R A) dil in barbiturate buffer, 0 125 M, at pH 7 4 ± 0 05, 0 0025 M Mg⁺⁺*
B 0 1 ml phage (1 × 10⁴ T2 particles) added to original reaction mixtures incubated *for 2 hours at 37° C*

Inactivation at varying phage concentration The standard inactivation test procedure was applied to study the influence of initial phage concentration on the course of inactivation Four different phage concentrations ranging from 10^4–10^7 particles per ml were used After incubation with serum, the reaction mixtures were diluted to give an expected number of about 200 plaques on the phage control plates

As seen in Fig 1, the proportion of inactivated T2 particles was found to be independent of the initial phage concentration

As the result could be influenced by reactivation due to dilution, the number of surviving particles from the highest phage concentration used was assayed immediately after dilution and also after standing for two hours at 37° C to allow postulated equilibrium conditions to be reached There was no significant difference between the assays

Inactivation at varying temperature With the aid of the standard inactivation procedure, the inactivation brought about by constant serum concentrations after four hours incubation at different temperatures was determined As shown in Fig 2, the inactivation reached a measurable level at 17° C while none was observed at 12° C With a serum concentration of 1 10, an optimum level of inactivation seemed to be obtained within the range of 28°–41° C After an incubation of 24 hours at 6–8–10–12–14° C, inactivation varying from 14–56 per cent was recorded for temperatures above 10° C with serum diluted 1 2 and 1 4 No inactivation was observed after 9 days incubation at +4° C

In the experiment illustrated in Fig 2 the concentration of serum

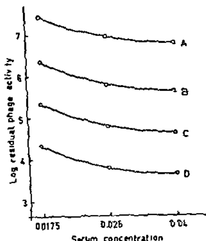


Fig 1
Inactivation of various concentrations of T2 by various concentrations of normal serum at 37° C

Abscissa Arithmet value of serum concentration on log scale Initial log phage activity A 7 36 B 6 35 C 5 30 D 4 33 Reaction mixture Total volume 1 ml 1 × 10⁴ T2 particles phage and serum (A D) dil in barbiturate buffer, 0 125 M at pH 7 4 ± 0 05 0 0025 M Mg⁺⁺ Reaction time 2 hours

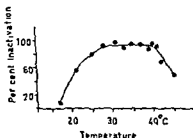


Fig 2
Inactivation of T2 by normal serum at various temperatures

Final serum dil 1 10 Reaction mixture Total volume 1 ml 1 × 10⁴ T2 particles phage and serum (A A) dil in barbiturate buffer, 0 125 M at pH 7 4 ± 0 05 0 0025 M Mg⁺⁺ Reaction time 4 hours

was chosen high enough to give a demonstrable inactivation at 17° C As the inactivation between 28–41° C reached the level of slow progress of the reaction, it was necessary to repeat the experiment using various serum dilutions to get a true picture of events The results are listed in Table 2 The inactivation capacity at different temperatures is expressed as the reciprocals of the dilutions giving 50 per cent inactivation

According to these experiments the inactivation rate at the 50 per cent level was found to increase with temperature up to 37° C At

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	Final serum dilution					
	1 5		1 25		1 40	
	A	B	A	B	A	B
10	72	97*				
20	45	52				
30	34	30	112	112	100	96
40	19	20				
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* Figure deriving from a single experiment, the other data represent the means of two identical experiments

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As seen in Fig 1, the proportion of inactivated T2 particles was found to be independent of the initial phage concentration

As the result could be influenced by reactivation due to dilution, the number of surviving particles from the highest phage concentration used was assayed immediately after dilution and also after standing for two hours at 37° C to allow postulated equilibrium conditions to be reached There was no significant difference between the assays

Inactivation at varying temperature With the aid of the standard inactivation procedure, the inactivation brought about by constant serum concentrations after four hours incubation at different temperatures was determined As shown in Fig 2, the inactivation reached a measurable level at 17° C while none was observed at 12° C With a serum concentration of 1 10, an optimum level of inactivation seemed to be obtained within the range of 28°–41° C *After an incubation of* 24 hours at 6–8–10–12–14° C, inactivation varying from 14–56 per cent was recorded for temperatures above 10° C with serum diluted 1 2 and 1 4 No inactivation was observed after 9 days incubation at +4° C

In the experiment illustrated in Fig 2 the concentration of serum

fluenced by many factors, the reproducibility was tested in experiment II Experiment III was set up to test the plaque size of the persistent fraction

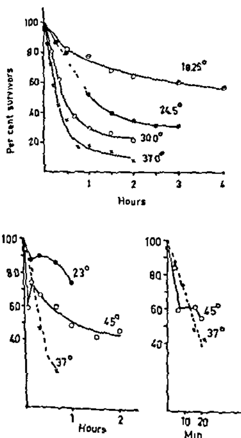


Fig 3
Time survivor curves of T2 at various temperatures and constant concentration of normal serum

Final serum dil 1 5 Reaction mixture Total volume 1 ml 1×10^{4} T2 particles, phage and serum (S S A) dil in barbiturate buffer, 0 125 M, at pH 7 4 ± 0 05, 0 0025 M Mg Each of the three figures is derived from a separate experiment In the bottom right fig inactivation was also followed at 41° The course of this curve was intermediate to those at 37° and 45°

All counting plates had *about* the same thickness (± 1 mm) If all plaques on a plate were not measured, plaques within an area chosen at random were determined

The plaques of the survivors were found to be smaller than the plaques on the control plates (Table 3) The probability of difference between the data obtained was *analysed* according to the conventional variance ratio test As to *experiment I*, all six mean plaque diameters were compared It could be demonstrated that there was a highly

temperatures above 37° C the rate appeared first to decrease gradually and then, at about 45° C, abruptly

The experiments thus seemed to indicate that the reaction has a very high temperature dependence at low temperatures, corresponding to a Q 10[1] of 7.4 at 17°–21° C. A second interesting observation was the retardation of the velocity above 37° C, especially the rapid drop at about 45° C.

TABLE 2

Effect of Temperature on the Inactivation of T2 by Normal Serum

Temperature (° C)	Reciprocal of serum dilution reducing initial phage activity to 50 per cent
23	6.4
29	25.4
37	35.9
41	34.2
43.5	31.2
45	23.0

Reaction mixture: Total volume 1 ml, 1 × 10[4] T2 particles phage and serum (R.A.) dil. in barbiturate buffer, 0.125 M, at pH 7.4 ± 0.05, 0.0025 M Mg^{++}. Incubation time 2 hours.

To investigate if the apparently decreasing reaction rate above 37° C could be attributed to heat inactivation of the phage inactivating principle, various dilutions of normal serum (S.S.A.) were heated at 37°, 40°, 44° and 45° C for two hours. The phage inactivating capacity was then tested according to the standard procedure at 37° C. The heating of serum at the temperatures indicated was not found to cause any significant reduction of the inactivating capacity.

The findings made further experiments necessary. The course of inactivation at various temperatures was followed at close time intervals as indicated in Fig. 3. It will be seen in the fig. that the high temperature dependence and the retardation of the reaction rate above 37° C indicated by the first experiments could be ascribed to deviations from the linear course of inactivation.

If only the initial rates were considered, the velocity was found to increase continously with temperature. At the temperature range 23°–30° C the increase in velocity corresponded to Q 10 = 1.96 and at 30°–37° C to Q 10 = 1.60. The mean of two determinations of the rate at 45° C gave Q 10 = 2.81 at the interval 37°–45° C.

Plaque size of surviving particles. In three time–survivor experiments the diameters of the plaques were measured. Experiment I (Table 3) was devoted to plaque size after incubation of the reaction mixtures at different temperatures. As plaque size is known to be in-

[1] The temperature coefficient calculated according to the van't Hoff equation

$$Q_{10} = \left(\frac{k_2}{k_1}\right)^{\frac{10}{t_2 - t_1}}$$

fluenced by many factors, the reproducibility was tested in experiment II Experiment III was set up to test the plaque size of the persistent fraction

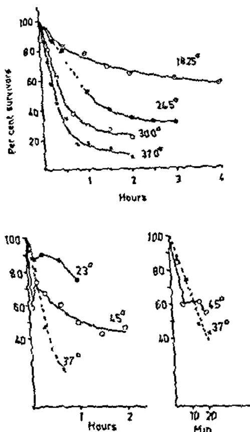


Fig 3

Time survivor curves of T2 at various temperatures and constant concentration of normal serum

Final serum dil 1 5 *Reaction mixture Total* volume 1 ml 1 × 10⁸ T2 particles phage and serum (S S A) dil in barbiturate buffer, 0 125 *M, at pH 7 4 ± 0 05,* 0 0025 M Mg *Each of the* three figures is derived from a separate experiment *In* the bottom right fig inactivation was also followed at 41° The course of this curve was intermediate to those at *37° and 47°*

All counting plates had about the same thickness (± 1 mm) If all plaques on a plate were *not* measured, plaques within an area chosen at random were determined

The plaques of the survivors were found to be smaller than the plaques on the control plates (Table 3) The probability of difference between the data obtained was analysed according to the conventional variance ratio test As to experiment I, *all* six mean plaque diameters were compared It c ** be demonstrated that there was a highly

TABI F 3

Size of Plaques Produced by Phage Particles Surviving Incubation with Normal Serum at Different Time and Temperature

Experiment	Reaction mixture	Temperature (°C)	Time (min)	Mean of plaque diam (mm)	Standard deviation (mm)	Number of plaques measured*	Per cent survivors
I	Phage + serum (final dil 1 5)	37 0	15	1 45	0 41	36	69
		37 0	60	1 29	0 41	36	42
		37 0	90	1 28	0 34	36	24
		24 0	90	0 85	0 33	36	42
		24 0	150	0 96	0 36	36	34
	Phage + broth	30 0	120	1 54	0 49	36	100
II	Phage + serum (final dil 1 5)	24 5	45	1 24	0 42	36	77
		24 5	90	1 19	0 37	20	47
	Phage + broth	37 0	120	1 63	0 38	36	100
III	Phage + serum (final dil 1 5–1 11)	37 0	120	1 46	0 39	138	11–17
	Phage + broth	37 0	120	1 57	0 30	80	100

Reaction mixture Total volume 1 ml 1×10^4 T2 particles phage and serum (S S A) dil in barbiturate buffer, 0 125 M, at pH 7 4 ± 0 05 0 0025 M Mg

Measurements made by a micrometer at low power magnification

* Deriving from duplicate platings

significant difference (variance ratio 17 06 $p < 0\ 0005$) between the observed diameters The six data were individually compared for differences at the 5 per cent level of significance according to *Hartley* as cited by *Snedecor* (1956) *It was found that the plaques formed by* phages surviving incubation in serum at 24° C for 90 and 150 minutes were significantly smaller than those of phages incubated at 37° C for 15, 60 and 90 minutes, the plaques obtained from the phage-serum mixtures incubated for 60 and 90 minutes being significantly smaller than those of the broth controls Of the values obtained in experiment II only the mean plaque diameters after incubation of phage + serum at 24 5° C for 45 minutes and of phage + broth at 37° C for 120 minutes were compared according to the conventional variance ratio test The difference in plaque size proved to be highly significant (variance ratio 16 77, $p < 0\ 0005$) In experiment III, the significance of the difference in mean plaque size of the survivors constituting the persistent fraction at 37° C and the controls was lower (variance ratio 4 06, $p < 0\ 05$)

Inactivation at varying hydrogen-ion concentration The rate of inactivation at varying pH was tested in a series of experiments using five different sera Barbiturate or Tris maleate buffers of different pH were used as diluents The hydrogen-ion concentration of the final reaction mixture was tested by electric pH-meter The degree of inactivation was calculated against controls of phage and heat-inactivated serum at the same dilution and pH The reaction mixtures and controls were treated according to the standard procedure

TABLE 4
Rate of Inactivation at Varying pH

pH	Velocity*
6 8	1 00
7 0	1 17
7 4	1 02
7 8	0 92

* Expressed [illegible]

The pH dependence was studied for pH 6–8 Within this range the reaction rate was found to be dependent on the pH in a moderate way, all sera exhibiting an optimum at pH 7 0 Table 4 shows the inactivation rate at pH 6 8–7 8

on [illegible]
wl [illegible]
re [illegible] were used as diluents in inactivation tests performed according to the standard procedure The result is given in

Table 5 where the effect on inactivation is expressed as the reciprocals of the serum dilutions inactivating 50 per cent of the phage particles. As will be seen, optimum inactivation occurred at 0.065 M NaCl, corresponding to a total molarity of 0.100 M.

TABLE 5

Effect of Various Salt Concentrations on the Inactivation of T2 by Normal Serum

Molarity			Reciprocal of serum dilution reducing initial phage activity to 50 per cent
NaCl	Tris maleate buffer	Total	
0.055	0.035	0.090	17.9
0.065	*0.035*	*0.100*	*29.0*
0.085	0.035	0.120	26.8
0.090	0.035	0.125	25.1
0.095	0.035	0.130	23.2
0.105	0.035	0.140	20.0
0.115	0.035	0.150	18.9
0.125	0.035	0.160	15.9
0.135	0.035	0.170	13.3
0.145	0.035	0.180	10.8

Reaction mixture: Total volume 1 ml. 1×10^4 T2 particles, phage and serum (A.O.) dil. in Tris (hydroxymethyl) aminomethane maleate (Tris maleate) buffer at pH 7.1 ± 0.1, 0.0025 M Mg^{++}. Incubated 2 hrs. at 37° C ± 0.3° C.

A similar experiment was set up using diluents consisting of varying concentrations of Tris-maleate and constant concentration of NaCl to see which factor was the most important, the concentration of NaCl or the total molarity. As shown in Table 6, optimum inactivation occurred at 0.100 M. As 0.100 was the lowest molarity tested, the experiment was repeated with buffer solutions containing 0.060 M NaCl and different concentrations of Tris-maleate to give total molarities varying around 0.100. Optimum conditions were achieved at 0.110 M.

TABLE 6

Effect of Molarity of Diluent on the Inactivation of T2 by Normal Serum

Molarity			Reciprocal of serum dilution reducing initial phage activity to 50 per cent
NaCl	Tris maleate buffer	Total	
0.0915	0.0085	0.100	35.0
0.0915	0.0285	0.120	32.3
0.0915	0.0335	0.125	30.3
0.0915	0.0385	0.130	28.2
0.0915	*0.0485*	*0.140*	*23.3*
0.0915	0.0585	0.150	19.3
0.0915	0.0885	0.180	14.7

Reaction mixture: Total volume 1 ml. 1×10^4 T2 particles, phage and serum (A.A.) dil. in Tris (hydroxymethyl) aminomethane maleate (Tris maleate) buffer at pH 7.1 ± 0.1, 0.0025 M Mg. *Incubated 2 hrs. at 37° C ± 0.3° C.*

DISCUSSION

If the inactivation of T2 by normal serum was merely the result of collisions between the participating molecules a temperature coefficient of about 1 5 might be expected The replacement of extraneous material on the receptor spots of the inhibited phage particles in crude lysates by the inactivating substances of normal serum (*Kallings* 1961) necessitates a higher energy of activation For comparison the neutralization of activated T2 particles by immune antibodies has a Q 10 of 1 4 (*Cann & Clark* 1954) contrasting to a Q 10 of about 2 for neutralization of native T2 (*Hershey* 1941 *Kalmanson et al* 1942)

Confined to the initial rates of inactivation of *T2 by normal serum* calculations of the temperature coefficient at various temperature levels resulted in a Q 10 of about 2 as reported above Thus the complex reaction of inactivation of T2 by normal serum seems to take place at about the same energy of activation as neutralization by immune serum This calls attention to the possible participation of a catalyst in the first mentioned reaction

As described above determination of the decline of phage activity at close time intervals revealed that the deviation from the linear course appeared at lower levels of inactivation at temperatures above and below 37° C than at 37° For example at 23° the constant rate seemed to be disturbed as early as after inactivation of the first 10 per cent of the initial phage activity The resemblance to the temperature characteristics of an enzyme reaction is conspicuous

Reflecting the complex chain of reactions there are two types of curves one smooth as at 37° C and one discontinuous particularly marked at 45° (Fig 3) The discontinuous progress of inactivation appears below as well as above the optimum temperature In the present stage there seems to be no satisfying explanation of the phenomenon Besides the steps leading to the final inactivation of the phage particles several antagonistic reactions probably with different temperature dependences have to be considered the activating effect of normal serum on inhibited phage particles in crude lysates the effect of preformed inhibitors to the phage inactivating serum principles and the effect of inhibitors produced during the course of inactivation As in typical enzyme reactions the last mentioned inhibitors may be the complex of virus particle—inactivating substances itself or breakdown products Though according to the *experiment described* above the inactivating effect of normal serum was not diminished by heating undiluted serum at the temperatures (<45° C) and periods used in the present study heat denaturation of factors in the reaction mixture may still occur

The contact with normal serum was found to diminish the plaque size of surviving phage particles The influence on plaque size was greater at 24 than at 37° At 37° this influence was more marked during

Table 5 where the effect on inactivation is expressed as the reciprocals of the serum dilutions inactivating 50 per cent of the phage particles. As will be seen, optimum inactivation occurred at 0.065 M NaCl, corresponding to a total molarity of 0.100 M.

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0.095	0.035	0.130	23.2
0.105	0.035	0.140	20.0
0.115	0.035	0.150	18.9
0.125	0.035	0.160	15.9
0.135	0.035	0.170	13.3
0.145	0.035	0.180	10.8

Reaction mixture. Total volume 1 ml; 1×10^4 T2 particles; phage and serum (A.O.) dil. in Tris (hydroxymethyl) aminomethane maleate (Tris maleate) buffer at pH 7.1 ± 0.1, 0.0025 M Mg^{++}. Incubated 2 hrs. at 37° C ± 0.3° C.

A similar experiment was set up using diluents consisting of varying concentrations of Tris-maleate and constant concentration of NaCl to see which factor was the most important, the concentration of NaCl or the total molarity. As shown in Table 6, optimum inactivation occurred at 0.100 M. As 0.100 was the lowest molarity tested, the experiment was repeated with buffer solutions containing 0.060 M NaCl and different concentrations of Tris-maleate to give total molarities varying around 0.100. Optimum conditions were achieved at 0.110 M.

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0.0915	0.0385	0.130	28.2
0.0915	0.0485	0.140	23.3
0.0915	0.0585	0.150	19.3
0.0915	0.0885	0.180	14.7

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REFERENCES

1. *Barlow, J. L., Van Vunakis, Helen & Levine, L.* Studies of the inactivation of [illegible]
2. [illegible]
3. [illegible] 41 233 319, 1941
4. *Jerne, N. K.* Bacteriophage inactivation by antiphage serum diluted in distilled water, Nature *169* 117-118, 1952
5. *Jerne, N. K. & Skovsted, L.* The rate of inactivation of bacteriophage T4r in specific anti serum I Salt effect II Cofactor Ann Inst Pasteur, *84* 73-89, 1953
6. [illegible]
7. [illegible] 1-12, 1942
8. *Snedecor, G. W.* Statistical methods, page 253 Iowa State College Press, Iowa, 5th ed., 1956

the linear progress of inactivation than at the level of retarded rate. In this connection *it is interesting to note that the inactivation at 24°* was arrested at a much earlier level. Whether these observations may be taken as evidence for the existence of incompletely inactivated phage particles by normal serum will be discussed in connection with reactivation experiments to be reported in a following paper.

The reaction rate was found to be moderately influenced by the pH and strongly influenced by the total molarity of the reaction medium. Optimum rate was achieved at pH 7.0 and at a molarity of 0.100–0.110, i.e. at a molarity below that of physiological diluents. *Barlow et al.* (1958) have reported optimum pH between 6.9 and 7.5 and a maximum inactivating activity of normal serum with a salt concentration between 0.126–0.150 M.

The present findings bring to mind the observation of *Jerne* (1952) and *Jerne & Skovsted* (1953), confirmed by *Cann & Clark* (1954), that physiological diluents inhibit phage neutralization by immune antibodies. They obtained optimum rate in 0.001 M NaCl.

As found in the reported experiments, the inactivation reaction seems to take place in an excess of inactivating principle. The activity of fresh phages added to an incubated phage-serum mixture declines at the same rate as the phages originally added. The inactivation of different phage concentrations proceeds in agreement with the percentage law.

Suppose that at excess of inactivating normal serum components, the uptake of these components follows a first order reaction. This implies that the pace of the inactivation process must be set by a subsequent reaction.

The assumption of an enzymatic nature of the pace-making reaction fits the experimental findings as do complement and Mg^{++} dependence.

SUMMARY

The inactivation of coli T2 phage by normal serum was found to take *place at excess of inactivating principle.*

The progress of inactivation at different temperatures corresponds to a Q 10 of about 2. Dependent on the temperature, the reaction seems to be inhibited after an initial linear course.

Contact with normal serum could influence the plaque size of the surviving T2 particles.

Optimum reaction rate was achieved at pH 7.0 and 0.100 M.

The results are discussed in relation to the findings reported in neutralization of phages by immune serum.

The characteristics of the inactivation process by normal serum have been found to resemble an enzymatic reaction in many respects.

cold nutrient broth and immediately plated according to the standard procedure. Two further reaction mixtures of each dilution were diluted 10^{-4} in prewarmed broth after the incubation and then kept at 37° C for 2 hours before being plated. To determine the initial virus activity and the spontaneous decrease both procedures were repeated with four phage broth controls. After dilution and immediate plating of the reaction mixtures containing a serum concentration of 1 25 28 per cent of the phages remained active whereas 25 per cent survived of those kept at 37° C after the dilution. The figures for the activity in the dilutions with serum concentration 1 38 were 59 and 63 per cent.

The degree of inactivation caused by the two serum concentrations used in this experiment was the same as had previously been found in experiments where the initial phage concentration was 10^4 infectious particles and the reaction mixtures only diluted 1 10 in physiological saline. The lack of evidence of reactivation in the present experiment agrees with the findings reported in connection with experiments indicating that the inactivation reaction conforms with the percentage law (*Kallings* 1961 b).

In preliminary experiments the dilution 1 10–1 100 in saline or distilled water of phage serum mixtures (serum B S, dil 1 20) incubated for 2 hours at 37° C caused no signs of reactivation nor did storing of the undiluted reaction mixture at + 4° C for 7 days.

In analogy with observations concerning the inactivation of bacterial and animal viruses by immune serum it might be assumed that the stability of the phage normal serum complex should increase with the reaction time. The reaction mixtures (serum S S A dil 1 17 and 1 20) were therefore incubated for only one hour in order to enhance the reactivation by dilution. Dilutions 1 10 1 100 1 500 and 1 1000 in saline and distilled water were plated after one hour at room temperature and after 6 days at + 4° C. Controls of phage and heat inactivated serum were included in the experiments. No significant difference in activity was found with increasing dilution or between the two diluents used when the dilutions were kept for one hour at room temperature before being plated. However storing at + 4° C for 6 days caused an increase in activity which was more evident when the reaction mixtures were diluted in distilled water (61 per cent average survival) than in saline (33 per cent average survival). The corresponding survival after one hour at room temperature was 25 per cent. The experimental procedure was the same as used in the experiment summarized in Table 1. In this experiment however the dilutions were only kept at room temperature for one hour before plating. Owing to the relative instability of the T2 phages in distilled water, room temperature was choosen instead of 37° C to secure reproducibility of the test results. As will be seen in Table 1 using serum A 6 activity increased with dilution as soon as after one hour at room temperature. The increase was greater in distilled water than in saline.

FROM THE NATIONAL BACTERIOLOGICAL LABORATORY, STOCKHOLM AND THE MICROBIOLOGICAL DEPARTMENTS OF THE MUNICIPAL LABORATORIES GOTHENBURG

STUDIES ON THE INACTIVATION OF BACTERIAL VIRUSES BY NORMAL HUMAN SERUM

3 *Reactivation of Inactivated Coli T2 Phages*[1]

By

LARS OLOF KALLINGS[2]

Received 12 i 1961

Studies on the kinetics of the reaction between coli T2r+ phages and normal serum have been presented in two previous papers (*Kallings* 1961). The reactivation experiments to be reported[3] in the present paper were performed in order to study the nature of the combination between the phage particle and the inactivating serum factors. Attempts have been made to reactivate coli T2r+ phages in reaction mixtures of phage and serum by dilution at various ionic strengths and pH, by treatment with a cation exchange resin to remove Mg^{++}, and with proteolytic enzymes.

As will be evident from the following experiments certain methods did not restore infectivity. These negative experiments have also been reported in detail with respect to their significance for the understanding of the forces uniting phage and serum factors.

MATERIAL AND METHODS

The material and methods were the same as described in a previous paper (*Kallings* 1961 a). High titer phage stocks were prepared by the agar layer technique yielding 10^{11-12} infectious particles per ml (*Swanstrom* & *Adams* 1951).

EXPERIMENTAL

Effect of Dilution in Broth, Saline and Distilled Water

About 7.5×10^9 T2 particles were incubated for two hours at 37° C in a serum (A.A. diluted 1:25 and 1:38 in barbiturate buffer). Duplicate portions of each concentration were then chilled, diluted 10^{-4} in

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[1] Aided by grants from the *Swedish Medical Research Council*.

[2] The skilful technical assistance of Mrs *Maud Lindberg* and Miss *Birgitta Sundisson* is gratefully acknowledged.

[3] A preliminary report was read at the 1959 Scandinavian Congress of Pathology and Microbiology. Publ. in Acta path. et microbiol. scandinav. Suppl. *144*, 207, 1961.

relative number of active phage particles was higher after dilution 1:100 in distilled water than in saline. The difference between the means of the survival was 9.6 per cent. The relative number of survivors after dilution 1:10 in saline was not lower than after dilution 1:100. It is interesting to note that the inactivation caused by serum B.W. (dil. 1:20) had reached the level of arrested rate (Fig. 1).

To sum up, under certain conditions the infectivity of phages inactivated by normal serum could be re-established to a limited extent by dilution. Prolonged storing of the inactivated phages in distilled water proved to be the most effective way (36 per cent reactivation). Under favourable conditions exposure to room temperature for one hour was found to increase the activity by about 10 per cent. The dilution effect seemed to be dependent on the duration of the preceding incubation of phage and serum as also on individual properties of different sera.

Effect of Dilution in Media of High Ionic Strength

In [illegible] ...ge particles and [illegible] *et al.* (1956) to [illegible] In four experiments, phages and normal serum were incubated and then diluted as described in Table 4. Three diluents were used: Michaelis buffer + 2 M NaCl according to Pillemer, 2 M NaCl and 0.15 M NaCl. The dilutions were kept at 37° C for one hour before plating. To prevent reunion when the dilutions were mixed with the isotonic plating media, part of each dilution was first kept at 56° C for 30 minutes to destroy the phage inactivating ability.

Preliminary experiments had first been performed to investigate if the mere heat denaturation of the serum factors could restore the infectivity of the inactivated phage particles. Reaction mixtures of phages and serum (A.A., B.S. and A.Ö., diluted 1:2.5 and 1:20) were incubated for one and two hours at 37° C and heated at 56° C for [illegible] ...e percentage of active particles was [illegible]
In [illegible] ...e diluted reaction mixtures did not [illegible] significant difference compared to the unheated dilutions. The activity was calculated according to the plaque count in heated and unheated phage controls.

As will be seen in Table 4, only an inconsiderable difference was found between the relative number of active phage particles in the three diluents of different molarity. Thus the difference between the means of the data for the reaction mixtures diluted in 2 M and 0.15 M NaCl was only 4.5 per cent.

Effect of Varying the Hydrogen Ion Concentration

Two sets of experiments were performed. In both of them, phage and serum were mixed and incubated at 37° C. In the first set the reaction mixtures were brought to different pH values by the cautious addition

TABLE 3

Dilution of Reaction Mixtures in Saline and Distilled Water

Serum	Dilution		Reaction mixture diluted in: I Saline 1:100			II Aq. dest. 1:100			III Saline 1:10		
			Native serum Plaque count†	Per cent survivors	Inactivated serum* Plaque count†	Native serum Plaque count†	Per cent survivors	Inactivated serum Plaque count†	Native serum Plaque count†	Per cent survivors	Inactivated serum Plaque count†
D	1:17	1	61.5	30.7	200.3	94.5	47.8	197.6	91.5	38.1	240.0
		2	68.0	33.9		77.0	39.0				
	1:25	1	99.5	49.7	200.3	99.5	50.4		125.0	52.1	
		2	90.0	44.9		114.5	57.9				
A	1:20	1	22.0	11.0	200.7	23.5	11.9	197.6	22.0	9.2	240.0
		2	21.5	10.7		17.0	8.6				
	1:33§	1	43.5	27.7	156.8	50.0	36.3	137.8	60.0	32.8	183.0
		2	53.0	33.8		65.5	47.5				

* Heated at 56° C for 30 minutes.
† Means of a duplicate experiment.
§ Values deriving from a separate duplicate experiment.

Reaction mixture: Serum D (A.O.) and A (B.W.) diluted in barbiturate buffer, 0.125 M, pH 7.4 ± 0.05, 0.0025 M Mg^{++}. I and II: about 1×10^5 T 2 particles per ml. III: about 1×10^4 T 2 particles per ml. Reaction mixtures incubated 1 hour at 37° C, diluted and plated immediately (1) or after 1 hour at 22° C (2). Plated according to the standard procedure.

relative number of active phage particles was higher after dilution 1 100 in distilled water than in saline The difference between the means of the survival was 9 6 per cent The relative number of survivors after dilution 1 10 in saline was not lower than after dilution 1 100 It is interesting to note that the inactivation caused by serum B W (dil 1 20) had reached the level of arrested rate (Fig 1)

To sum up, under certain conditions the infectivity of phages inactivated by normal serum could be re-established to a limited extent by dilution Prolonged storing of the inactivated phages in distilled water proved to be the most effective way (36 per cent reactivation) Under favourable conditions exposure to room temperature for one hour was found to increase the activity by about 10 per cent The dilution effect seemed to be dependent on the duration of the preceding incubation of phage and serum as also on individual properties of different sera

Effect of Dilution in Media of High Ionic Strength

In attempts at splitting the union between the phage particles and the inactivating serum factors, the method of *Pillemer et al* (1956) to eluate properdin from the PZ complex was adopted In four experiments, phages and normal serum were incubated and then diluted as described in Table 4 Three diluents were used, Michaelis buffer + 2 M NaCl according to Pillemer, 2 M NaCl and 0 15 M NaCl The dilutions were kept at 37° C for one hour before plating To prevent reunion when the dilutions were mixed with the isotonic plating media, part of each dilution was first kept at 56° C for 30 minutes to destroy the phage inactivating ability



As will be seen in Table 4, only an inconsiderable difference was found between the relative number of active phage particles in the three diluents of different molarity Thus, the difference between the means of the data for the reaction mixtures diluted in 2 M and 0 15 M NaCl was only 4 5 per cent

Effect of Varying the Hydrogen Ion Concentration

Two sets of experiments were performed In both of them, phage and serum were mixed and incubated at 37° C In the first set the reaction mixtures were brought to different pH values by the cautious addition

TABLE 4

Per cent Phage Activity after the Dilution of a Reaction Mixture in Iso and Hypertonic Saline

Reaction mixture			Dilution of the reaction mixture 1:26 in							
Serum	Dilution	Incubation time at 37° C in hours	3.6 ml Michaelis buffer pH 7.4 + 1.4 ml 2 M NaCl		2 M NaCl		0.15 M NaCl		0.15 M NaCl No incubation	
			Incubated 1 hour at 37°C before plating							
			I	II	I	II	I	II	I	II
I M	1:5	2	39.2	33.3	45.6	38.6	42.7	41.5		
S S A	1:5	2	13.7	16.7	19.7	23.3	14.3	13.7	12.5	
S S A	1:20	1	35.5	32.3	33.3	35.0	28.3	30.5	30.3	21.8
R A	1:17	1			57.2	54.5	57.4	42.7	61.9	57.1
Means					39.0	37.9	35.7	32.1		
					38.4		33.9			

I Plated without previous heating
II Heated at 56° C for 30 min before plating

Reaction mixtures 1–2 × 10^4 T 2 particles and serum diluted in barbiturate buffer, 0.125 M, pH 7.4 ± 0.05 Mg^{++} added to 0.0025 M. Plating 1 ml dilution mixed with 3.5 ml melted agar containing approx 1.5 × 10^8 coli B cells 2 ml poured as top layer. Phage controls with *inactivated serum treated as the reaction mixtures*. *All* experiments performed in duplicate or triplicate

of 0 1 n HCl or 0 1 n NaOH, then stored at 37° C or 22° C for 30 or 60 minutes respectively, chilled, neutralized, diluted 1 10 in ice cold saline and plated according to the standard procedure The pH values were determined with an electrical pH meter The effect was tested at six pH values ranging between 9 2 and 4 1, the latter being the isoelectric point of the T2 particles The two different sera used (A A and B S) were diluted 1 2 5 and 1 25 respectively At each pH value the phage activity was calculated according to the plaque count of controls consisting of phages and heat inactivated sera, set up and treated in the same way as the reaction mixtures The pH variation was not found to increase the number of active phage particles in the reaction mixtures

In two other experiments reaction mixtures incubated for 30 and 60 minutes were chilled, diluted 1 10 in cold Tris maleate buffers of different pH, stored one hour and then diluted 1 10 in Tris maleate buffer at pH 7 0 before being plated To reduce the loss of infectivity due to the direct pH effect on the virus particles a high serum concentration was used (1 2) and the incubated reaction mixtures were kept at + 4° C the whole time before plating As in the above experiments, controls with inactivated serum were included The experiments were performed in triplicate Only the relative plaque count after dilution at pH 9 5 of the reaction mixture incubated for 30 minutes seemed to be a little higher (7 per cent) than the corresponding figure after dilution at pH 7 0 (Table 5)

TABLE 5

Dilution at Various pH Values of a Phage Serum Mixture Incubated for 30 and 60 Minutes at 37° C

pH of dil ent	Per cent survivors	
	30 min	60 min
4	29 2	30 1
5	30 3	30 1
6	34 9	37 1
7	36 4	37 6
8	35 4	37 1
8 5	36 9	29 6
9	37 4	33 9
9 5	44 1	31 2

Reaction mixture serum (S 6 A) diluted 1 2 in Tris (hydroxymethyl) amino methane maleate buffer pH 7 0 ± 0 05 0 125 M about 10^5 T 2 particles per ml Mg added to 0 0025 M

Owing to the scatter of the data obtained above, the procedure was repeated in two further triplicate experiments using another serum diluted 1 10 As in the previous experiments no definite reactivating effect could be demonstrated

Effect of Removing Mg^{++} from the Incubated Mixture of Phage and Serum

As was reported by *Barlow et al* (1958) passing a serum through a resin removing divalent cations or chelating the cations by ethylenediamine tetraacetate depletes the serum of inactivating capacity The activity is restored by the addition of Mg^{++} but not by the addition of Ca^{++} These findings could be confirmed by the present author (unpublished experiments). Thus, magnesium ions seem to participate in the reaction between the phage particles and the inactivating serum factors The following experiments were designed to investigate if attempts to remove Mg^{++} from the complex could cause reactivation

A resin of the carboxylic acid type was used (Amberlite IRC-50) Before use it was converted from the hydrogen to the sodium cycle by the addition of NaOH and neutralized by washing in 0 15 M saline

A portion of a reaction mixture of about 1×10^4 T2 particles per ml and serum (A A, dil 1:10) was incubated for 25 minutes at 37° C, chilled in ice-water and diluted 1.10 in cold barbiturate buffer and another portion was heated at 56° C for 30 minutes The mixtures were then transferred to dialyzer tubes which were placed in a beaker containing the resin suspended in serum heated at 56° C for 30 minutes and diluted 1·10 in barbiturate buffer Controls of phage and heated serum were treated similarly The beaker was left at room temperature overnight The resin was changed after one hour Next day the reaction mixtures and controls were dealt with according to the standard procedure The resin treatment was found to reduce the magnesium content of the reaction mixtures so that no magnesium could be detected with an organic reagent ((4-[p-nitrophenylazo]-1-naphtol), *Johnson* 1958) sensitive to 1 part magnesium in 5 million parts solution The relative phage activity was found to be about the same whether the reaction mixtures were heated or only diluted before treatment with the resin The mean activity of three determinations was 69 8 per cent which should be compared with the values obtained in the following experiment

A reaction mixture was made up incubated and chilled as indicated above In this experiment the resin was put directly into tubes containing the reaction mixture These tubes were incubated at 37° C for 25 minutes The inactivation after the initial incubation period was determined in untreated samples Controls of phage and heated serum were treated correspondingly The direct contact with resin was found to reduce the virus titer of the controls by an average of 18 per cent After the incubation of the phage-serum mixture with resin the mean activity in six samples treated separately was 74 0 per cent, practically the same as found in the untreated reaction mixtures (75 4 per cent)

The procedure was repeated in a triplicate experiment with the same dilution of serum (A A) and incubation time and with another serum (A 0) dil 1 10 in

cubated with T2 for 30 minutes The relative number of active phages was 65 7 without and 66 2 with resin treatment for the first serum 65 2 and 61 2 for the second serum tested)

Attempts to remove Mg^{+} from the complex of phage and inactivating serum factors thus did not prove to increase the number of infectious particles

Effect of Treatment with Proteolytic Enzymes

It is evident from the preceding experiments that the reaction leading to inactivation of phages by normal serum was not easily reversed Therefore, the effect of methods able to break comparatively complex unions was tested As the complement and other inactivating normal serum factors are known or supposed to be protein in nature, the effect of three enzymes hydrolysing —CO—NH—links (trypsin papain and ficin) was studied

Trypsin Trypsin (Difco 1 250) 1 g per 100 ml phosphate buffer was used Two experiments were performed In the first (I) phage suspension and serum (B S dil 1 20 in barbiturate buffer) were mixed and incubated for two hours at 37° C The reaction mixture was then heated at 56° C for 30 minutes to stop further inactivation and divided in two portions To the one of them 0 25 ml of the trypsin solution per ml was added and to the other 0 25 ml barbiturate buffer per ml Both portions were then incubated for 30 minutes at 37° C, diluted 1 10 in cold 0 15 M saline and plated according to the standard procedure

In a second experiment (II) phage suspension and another serum (A O dil 1 10 in barbiturate buffer) were mixed and incubated for two hours at 37° C, chilled and divided in two portions The first portion was diluted 1 10 in 0 15 M saline and plated To the second 0 2 ml trypsin solution per ml was added This portion was incubated for 10 minutes at 37° C before diluting and plating as above

TABLE 6
Effect of Treating the Phage Serum Mixture with Trypsin

Experiment		Reaction mixture		Phage control
		Plaque count*	Per cent activity	Plaque count*
I	Treated	114 0	51 6	221 0
	Untreated	92 5	38 1	242 5
II	Treated	54 5	38 1	143 0
	Untreated	33 5	27.3	122 5

* Means of [illegible]

[illegible]

Effect of Removing Mg^{++} from the Incubated Mixture of Phage and Serum

As was reported by *Barlow et al* (1958) passing a serum through a resin removing divalent cations or chelating the cations by ethylenediamine tetraacetate depletes the serum of inactivating capacity The activity is restored by the addition of Mg^{++} but not by the addition of Ca^{++} These findings could be confirmed by the present author (unpublished experiments) Thus, magnesium ions seem to participate in the reaction between the phage particles and the inactivating serum factors The following experiments were designed to investigate if attempts to remove Mg^{++} from the complex could cause reactivation

A resin of the carboxylic acid type was used (Amberlite IRC-50) Before use it was converted from the hydrogen to the sodium cycle by the addition of NaOH and neutralized by washing in 0 15 M saline

A portion of a reaction mixture of about 1×10^4 T2 particles per ml and serum (A A , dil 1 10) was incubated for 25 minutes at 37° C, chilled in ice-water and diluted 1:10 in cold barbiturate buffer and another portion was heated at 56° C for 30 minutes The mixtures were then transferred to dialyzer tubes which were placed in a beaker containing the resin suspended in serum heated at 56° C for 30 minutes and diluted 1 10 in barbiturate buffer Controls of phage and heated serum were treated similarly The beaker was left at room temperature overnight The resin was changed after one hour Next day the reaction mixtures and controls were dealt with according to the standard procedure The resin treatment was found to reduce the magnesium content of the reaction mixtures so that no magnesium could be detected with an organic reagent ((4-[p-nitrophenylazo]-1-naphtol), *Johnson* 1958) sensitive to 1 part magnesium in 5 million parts solution The relative phage activity was found to be about the same whether the reaction mixtures were heated or only diluted before treatment with the resin The mean activity of three determinations was 69 8 per cent which should be compared with the values obtained in the following experiment

A reaction mixture was made up, incubated and chilled as indicated above In this experiment the resin was put directly into tubes containing the reaction mixture These tubes were incubated at 37° C for 25 minutes The inactivation after the initial incubation period was determined in untreated samples Controls of phage and heated serum were treated correspondingly The direct contact with resin was found to reduce the virus titer of the controls by an average of 18 per cent After the incubation of the phage serum mixture with resin the mean activity in six samples treated separately was 74 0 per cent, practically the same as found in the untreated reaction mixtures (75 4 per cent)

The procedure was repeated in a triplicate experiment with the same dilution of serum (A A) and incubation time and with another serum (A O dil 1 10) in

percental activity was increased (9 2 per cent) after treatment with the activated papain

TABLE 8

Effect of Treating the Phage Serum Mixture with Papain

Treatment	Reaction mixture		Phage control Plaque count*
	Plaque count*	Per cent activity	
Activated papain	44 4	30 4	146 3
Non activated papain	40 3	22 2	181 8
	29 8	21 2	140 5

* Means of 3 duplicate experiments

Reaction mixture approx 9 × 10⁹ T 2 *particles per ml and serum* (A 6) diluted 1 10 in barbiturate buffer, 0 125 M pH 7 4 + 0 05 Mg⁺ added to 0 0025 M Phage control Equal parts of phage suspension and nutrient broth Papain added to 0 002 0 2 *per cent*

Ficin Freshly prepared one per cent aqueous solution of ficin (Mann) was used Reaction mixtures and controls were adjusted to pH 6 2 with 0 1 n HCl after the addition of the ficin solution

In two duplicate experiments T2 phages and serum (A O , dil 1 10) were incubated for two hours at 37° C One portion of the reaction mixture was treated with 0 25 ml ficin solution per ml (37° C, 10 min) The other portion was placed in icewater, 0 25 ml barbiturate buffer per ml added and pH adjusted to 6 2 Phage controls in broth were treated correspondingly All mixtures were then diluted and plated according to the standard method After treatment with ficin, 44 8 and 44 0 per cent of the phages were found to be active compared to 31 9 and 31 1 per cent in the untreated mixtures—thus a difference of 12 9 per cent in both experiments

In further experiments of this type the difference between the relative plaque count of treated and untreated mixtures was found to increase considerably if the reaction period between phage and serum was shorter or serum further diluted To examine this, T2 phages and serum (B S) diluted 1 40 were incubated at 37° C for 20 hours At the time intervals listed in Fig 2, samples were treated with ficin as in the above experiments The decrease in virus activity in untreated samples was determined at the same time Barbiturate buffer was added instead of ficin solution in these control samples and the pH adjusted to 6 2 Part of the control samples was chilled and diluted immediately and part was incubated for 10 minutes as the ficin treated mixtures The difference between these two steps was later found to be negligible The inactivation was calculated according to the plaque counts of ficin treated and untreated phage controls with serum heated at 56° C for 30 minutes As shown in the fig the difference between treated and untreated reaction mixtures was 34 per cent after one hour, 24 per cent after two hours and so on decreasing towards zero with increasing reaction time

The results of the two experiments are presented in Table 6. The treatment with trypsin was found to increase the number of active phages. The difference between the activity in treated and untreated mixtures was 13.5 and 10.8 per cent.

Papain. Papain 1:350 (Merck) was used in 2 per cent aqueous solution. The solution was made up according to *Kalmansson & Bronfenbrenner* (1943). Before use it was taken from the refrigerator and activated by the addition of 0.1 ml of 16 per cent cysteine hydrochloride per ml. The pH was adjusted to 7.4 and the mixture incubated at 37° C for 30 minutes (*Rosenheim* 1937).

The resistance of the phage particles towards the papain preparations was first examined as preliminary experiments had revealed a considerable decline of activity in treated phage controls. Different dilutions of activated and non-activated papain were added to a series of tubes containing one ml phage suspension preincubated for two hours at 37° C. After the addition of the enzyme the suspensions were incubated for 10 minutes at 37° C, diluted 1:10 in 0.15 M saline and plated according to the standard procedure. As is shown in Table 7 the titer of infective particles decreased in relation to the amount of papain added. A loss of infectivity was caused by activated as well as by non-activated papain. The stock suspension of virus used in this experiment had been stored in the refrigerator for about 6 months. In the following experiments a fresh lysate (1–2 weeks old) was used. As mentioned in a previous paper (*Kallings* 1961 a) in connection with the activation of inhibited phage particles in crude lysates, papain treatment of this fresh lysate was found to increase the phage activity by an average of 12.5 per cent. The same phenomenon was observed after treating a fresh lysate with trypsin. Ficin was only tested on old lysates.

TABLE 7

Effect of Papain on the Infectivity of the Phage Particles

Percental concentration of papain in the phage suspension	Plaque count*	
	Activated papain	Non-activated papain
0.2	91	54
0.1	134	119
0.02	149	162
0.002	156	157
–	175	175

* Means of a duplicate experiment.
Phage suspension: Approx. 9 × 10³ T 2 particles in 0.5 ml barbiturate buffer 0.125 M, pH 7.4 ± 0.05 + 0.5 ml nutrient broth. Mg++ added to 0.0025 M.

The action of papain on the union between phage and inactivating serum factors was tested in three duplicate experiments. Phage suspension and serum (A.O., dil. 1:10) were incubated at 37° C for two hours. The effect of the different papain concentrations given in Table 7 was examined. The mixtures were incubated for 10 or 30 minutes before dilution 1:10 in 0.15 M saline and plating according to the standard procedure. As the various papain concentrations gave about the same relative number of active phages, the results in Table 8 are given as the means of the observed plaque counts. As will be seen the

In the reported experiments the virus activity in the reaction mixtures was related to the activity in controls treated in the same way For instance activation by dilution in distilled water of phages inhibited by bacterial products (*Sagik* 1951) should be revealed by the controls Therefore the increase of the activity observed seems to be due to an actual reactivation process and not to the liberation of particles already inhibited in the original virus suspension

Methods able to cause molecular re arrangement were found more effective to reverse the reaction than methods able to break physical bonds Thus digestion of the complex by proteolytic enzymes always increased the number of active particles though only to a certain extent limited by the phage serum reaction time (Fig 2) The effect of methods apt to change equilibrium and electrostatic charge was found to be irregular and dependent on the individual properties of certain sera Prolonged storage in distilled water appeared to be the most effective of these procedures If the factors in the complex could be easily dissociated a shift in the equilibrium by dilution should give a quantitative release of free phages from the complex according to the law of mass action No consistent relation could however be demonstrated between the activity and the degree of dilution It is worthy of note that a moderately high salinity (0 6 M) enough to eluate properdin from zymosan (*Pillemer et al* 1956) did not restore the activity of the inactivated phages nor did dilution in 2 M saline

In contrast to the present negative result of treating the inactivated phages by a cation exchange resin *Wedgwood et al* (1956) found that the inhibition of Newcastle Disease Virus by normal serum was reversible to a certain extent by this treatment

The reported findings point to a firm union between the phage particle and the inactivating serum factors The tendency towards decreasing reactivation with increasing phage serum reaction time and serum concentration reflects a reaction proceeding to irreversibility The relation between the degree of reactivation and the period of preceding contact with serum is especially evident in the experiment shown in Fig 2 where the fraction of phages recovered after ficin treatment gradually decreases For comparison with specific antiphage serum digestion with papain was found to cause complete reactivation not limited by the length of contact of phage with antibody prior to treatment while if the phage was overneutralized it could not be reactivated (*Kalmanson & Bronfenbrenner* 1943)

With regard to the non specific character of the inactivating principle in normal serum the demonstrated stability of th com lex not

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im ization is moderately dissociable by dilution A plausible inter

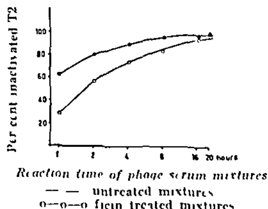


Fig 2

Increase in the number of infectious particles after ficin treatment of a phage—serum mixture incubated for various periods at 37° C

Reaction time given on log scale —Reaction mixture Serum (B S) diluted 1 40 in barbiturate buffer, 0 125 M, pH 7 4 ± 0 05 approx 1 × 10⁴ T 2 particles per ml Mg^{++} added to 0 0025 M

The experiment was repeated with consistent results when using another serum

The addition of trypsin, papain and ficin to serum was found to completely abolish the inactivating capacity of serum, due either to digestion of the inactivating serum factors or to non-specific inhibition of the reaction as seen after the addition of various proteins (*Kallings* to be published)

DISCUSSION

As shown by the present experiments, the number of phage particles remaining active after contact with normal serum may be increased under certain conditions The first question to be discussed is if this increase is caused by the re-establishment of the infectivity of separate or aggregated particles inactivated by serum factors, the dissociation of aggregates of native phages or by the activation of particles inhibited in some other way

Specific aggregation is known to occur with antiphage serum (*Schlesinger* 1933) High concentration of phage is however necessary to give a visible precipitate *Burnet* (1933) observed no reaction unless the original titer was 2 × 10^9 or higher Depending upon the size of the particles about 10^{11} 10^{12} phage particles per ml seem to be required for a visible reaction (*Merrill* 1936) Later *Hershey et al* (1943) found that if the concentration of phage exceeds *10^9* plaque-forming units per ml, the velocity of the reaction with antibody increases decisively which suggests specific aggregation of the phage If a corresponding phenomenon occurs with normal serum, it was not likely to appear in the present experiments as the suspensions were mostly diluted to 10^4 plaque-forming units before use

14 *Sagik B P* A specific reversible inhibition of bacteriophage T2 J Bacteriol 68 430 436 1954
15 *Schlesinger M* Die direkte nephelometrische Erfassung hoher Bakteriophagen konzentrationen in einem Medium mith geringer eigener Lichtstreuung Z Hyg *114* 746 753 1933
16 *Swanstrom M & Adams M H* Agar layer method for production of high titer phage stocks Proc Soc Exptl Biol Med *78* 372 375 1951
17 *Wedgwood R J Ginsberg H S & Pillemer L* The properdin system and immunity VI The inactivation of Newcastle Disease Virus by the properdin system J Exp Med *104* 707 725 1956

pretation seems to be just the imperfect specificity of this early form of antibody

The stability of the phage-normal serum complex seems to justify that the observed neutralization of the infectivity of phages after contact with normal serum may be characterized as an inactivation reaction

SUMMARY

Attempts have been made to reverse the inactivation process by dilution at various ionic strengths and pH values, by the exclusion of Mg^{++} and by digestion with proteolytic enzymes The complex between phage and the inactivating factors in normal serum proved to be of considerable stability Under certain conditions dilution, especially in distilled water, gave a measurable reactivation Only treatment with proteolytic enzymes caused a regular although not complete dissociation of the union After prolonged contact between phage and normal serum the reaction seemed to be irreversible The effect of normal serum on coli T2 phages may thus be looked upon as a true inactivation process

REFERENCES

1. *Barlow J L Van Vunakis Helen & Levine L* Studies of the inactivation of phage by the properdin system I Evidence for complement properdin and magnesium requirements J Immunol *80* 339 348 1958
2. *Burnet F M* Specific agglutination of bacteriophage particles Brit J Exptl Pathol *14* 302 308 1933
3. *Burnet F M Keogh E V & Lush D* Immunological reactions of filterable viruses Australian J Exptl Biol Med Sci *15* 227 368 1937
4. *Hershey A D* Experiments with bacteriophage supporting the lattice hypothesis J Immunol 47 77–87 1943
5. *Hershey A D Kalmanson G & Bronfenbrenner J* Quantitative methods in the study of the phage–antiphage reaction J Immunol *46* 267 279 1943
6. *Jerne N K & Avegno Perla* The development of the phage inactivating properties of serum during the course of specific immunization of an animal Reversible and irreversible inactivation J Immunol *76* 200 208 1956
7. *Johnsson W C* ed Organic reagents for metals Vol I 5th ed p 98 Hopkins & Williams Essex 1955
8. *Kallings L O* Studies on the inactivation of bacterial viruses by normal human serum I Kinetics of the coli T2 phage inactivation at various serum concentrations Acta path et microbiol scandinav *51* 38J 1961 a
9. *Kallings L O* Studies on the inactivation of bacterial viruses by normal human serum II Effect of phage concentration temperature pH and molarity on the kinetics of the coli T2 phage inactivation Acta path et microbiol scandinav *51* 71 1961 b
10. *Kalmanson G M & Bronfenbrenner J* Restoration of activity of neutralised biologic agents by removal of the antibody with papain J Immunol *47* 387 407 1943
11. *Merrill M H* The mass factor in immunological studies upon viruses J Immunol *30* 169 184 1936
12. *Pillemer L Blum Lucia Lepow I H Wurz Leona & Todd E W* The properdin system and immunity III The zymosan assay of properdin J Exp Med *103* 1–13 1956
13. *Rosenheim A H* The action of enzymes on antibodies Biochem J *31* 54 71 1937

EXPERIMENTAL

Stability at 37° C and below

The experiments under this heading were mainly performed to determine the temperatures and periods appropriate to preserve the inactivating potency and to investigate the decay of activity likely to occur in sera standing at room temperature and incubated at 37° C the reaction temperature generally used

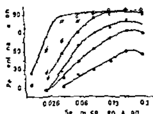


Fig 1

Decay of phage inactivating capacity of serum incubated at 37° C
Curves from left to right Serum unheated heated for 0 5 1 5 2 3 5 4 and 5 days Details are given in Table 1 Abscissa Arithmetical values of serum concentrations on a log scale

TABLE 1

Decrease of Phage Inactivating Capacity after Incubation at 37° C

Time	Reciprocal of serum titre inactivating 50% of the plaques	Serum activity in %
	44 1	100 0
12 hours	38 5	87.3
24	32 8	74 4
36	28 6	64 9
48	24 0	54 4
60 "	21 7	49 2
72	19 6	44 4
84	17 9	40 6
4 days	11 9	27 0
5	4 4	10 0

Reaction mixture Total volume 1 ml about 1 × 10³ T 2 particles phage and serum (1 A d) in barbiturate buffer 0 125 M at pH 7 4 + 0 05 0 0025 M Mg In cubated at 37° C for 2 hours Duplicate experiment

37° C The inactivating capacity was found to be unaltered after incubation of four different sera for 8 12 hours at 37 ± 0 3° C The sera were incubated undiluted After about 12 hours the inactivating capacity gradually diminished The decrease became first evident at low serum concentrations (Fig 1) As will be seen in this figure illustrating the effect of exposure to 37° C from 12 hours to 5 days the shape of the inactivation serum concentration curve changed with increasing incubation time of serum The typical curve with a steep slope at

STUDIES ON THE INACTIVATION OF BACTERIAL VIRUSES BY NORMAL HUMAN SERUM[1]

4 *Decay of the Coli T2 Phage Inactivating Capacity at Various Temperatures*[2]

By

LARS OLOF KALLINGS[3]

Received 16 ii 61

An extended knowledge of the thermostability of the virus neutralizing capacity of normal serum is important from several aspects for the practical handling of fresh serum, for the methods used to preserve or destroy the neutralizing ability, to estimate the deterioration of serum activity during various experimental conditions, and to elucidate the nature of the serum factors participating in the virus inactivation reaction

The inactivating property of normal serum on coli T2 bacteriophages is known to be thermolabile *Van Vunakis et al* (1956) found the inactivating effect to be destroyed by heating fresh serum at 56° C for 15 minutes In the present paper a detailed study will be reported on the stability to temperatures ranging between — 60° and + 80° C Evidence is presented for the appearance of a virus inhibiting effect when serum is heated at temperatures between about 54° 70° C The findings are discussed in relation to the thermostability of the complement factors, properdin and conventional specific antibodies

MATERIAL AND METHODS

The material and methods were described in previous papers (*Kallings* 1961 a c) Sterile sera were placed in a waterbath refrigerator or freezing box for various periods at the temperatures listed below diluted in 1 5 fold steps and tested for coli phage (T 2r) neutralizing activity according to a standard inactivation procedure (*Kallings* 1961 a) The serum dilutions mentioned below refer to the final dilutions in the reaction mixtures if not otherwise is stated All experiments were performed with sterile precautions

1 Aided by grants from the *Swedish Medical Research Council*

2 A preliminary report was read at the 1959 Scandinavian Congress of Pathology and Microbiology Published in Acta path et microbiol scandinav Suppl 144 207 1961

3 The skilful technical assistance of Mrs *Maud Lindberg* and Miss *Birgitta Sundosson* is gratefully acknowledged

modified A time—survival experiment was performed to facilitate the understanding of this phenomenon A preliminary experiment had first to be done Two lots of the same serum (A I A) a fresh one and one incubated undiluted at 37° C for three days were diluted in 1 5 fold steps and tested for phage neutralizing activity (two hours incubation at 37° C) The neutralizing potency of the serum lot pre incubated at 37° C was found to be decreased by 60 per cent of the original activity On the basis of this test two serum dilutions were selected for the time experiment causing A a neutralization rapidly reaching the level of persistent fraction and C a reaction somewhat slower Four mixtures of phage and serum were made up containing per ml A 0 065 ml fresh serum B 0 065 ml serum incubated undiluted at 37° C for three days C 0 045 ml fresh serum and D 0 045 ml serum incubated as B The mixtures were divided into 1 ml aliquots and were placed in a waterbath at 37° C At the intervals indicated in Fig 2 duplicate aliquots of each mixture were tested for remaining phage activity according to the standard procedure It was revealed by this experiment that the inactivation by pre incubated serum in comparison to that by fresh serum was characterized by a prolonged initial lag period and an earlier deviation from the linear phase to the retarded reaction rate

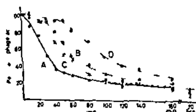


Fig 2

Time—survival curves of two concentrations of a serum incubated undiluted at 37° C for 3 days

Procedure and significance of symbols are indicated in the text Remaining complement activity in the serum lot incubated at 37° C for 3 days 89.3 50 per cent hemolytic units

2) C The stability of six different sera was tested at 22 ± 0 3 ° C The activity was found to be unchanged after about 30 hours incubation and to be reduced to 50 per cent after 4 5 days The findings are illustrated by two sera in Table 3 where the increasing serum concentrations necessary to inactivate 50 per cent of the phages are listed The progress of inactivation at different serum concentrations was influenced by the exposure of undiluted serum to 22° C in the same way as described for sera pre incubated at 37° C

3) C Four different sera were placed at 4 ± 0 5° C for varying periods The …

Table 4) H … ays

the inactivat … of

… for increasing periods of time at

high serum dilutions rapidly bending to the almost constant level of inactivation caused by the persistent fraction, gradually flattened out, the phase of persistence appearing at lower and lower levels of inactivation. The drop in inactivation at high serum concentrations demonstrated in the figure was found to recur in different sera after incubation of serum at various temperatures. In the experiment shown in the figure the reciprocal of the serum dilution able to inactivate 50 per cent of the phages after different periods of pre-incubation was calculated to express the decay of the inactivating property (Table 1). As shown in the table the initial activity was reduced to 50 per cent after about 60 hours. The 50 per cent decay appeared after about 48 hours in another serum.

The stability under the ordinary conditions of the inactivation test was also examined, i.e. the stability when sera were diluted prior to incubation at 37° C. Two different sera were first serially diluted in barbiturate buffer according to the standard inactivation test and then placed at 37° C for varying periods before phage was added. The mixtures of phage and serum were kept at 37° C for one or four hours. As seen in Table 2 diluted serum seemed to be more thermolabile than undiluted. The inactivating capacity of diluted serum was found to be unchanged after about 4 hours. A decrease of 50 per cent was reached as early as after 6–8 hours incubation.

TABLE 2

Decrease of Phage Inactivating Capacity after Incubation of Dilutions of two Sera at 37° C

Hours	Serum I: Reciprocal of serum dil. inactivating 50 % of the phages	Serum I: Serum activity in %	Serum II: Reciprocal of serum dil. inactivating 50 % of the phages	Serum II: Serum activity in %
	14.5	100.0		
1	13.9	95.9	46.1	100.0
2	14.9	102.8	45.9	99.6
4			45.5	98.7
6	11.2	77.2	33.8	73.3
8	3.3	22.8	21.3	46.2
10				

Reaction mixture: Total volume 1 ml; about 1×10^4 T 2 particles, phage and serum dil. in barbiturate buffer 0.125 M at pH 7.4 + 0.05–0.0025 M Mg. Incubated at 37° C: serum I (A. I. A.) for 1 hour, serum II (A. A.) for 4 hours. Duplicate experiment.

The reported findings indicate not only that the virus inactivating power of normal serum decreased during prolonged exposure to 37° C but also that the neutralization caused by such serum was qualitatively

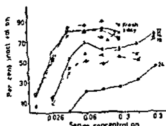


Fig 3
Decay of phage inactivating capacity of serum stored at +4° C for 1 24 days
Abscissa Arithmetical values of serum concentrations on a log scale
Details are given in Table 4 serum II

−30° C The inactivating capacity of two sera stored at — 30 ± 1° C was diminished after about two months (80 and 87 per cent of the initial activity expressed as the reciprocal of the dilution able to inactivate 50 per cent of the phages) The influence of storage at — 30° C on the inactivation at different serum concentrations was similar to that observed after storage at +4° C

—60° C Sera were stored in rubber—stoppered tubes in a dry ice box As the tubes were not sealed the influence of CO_2 on the phage inactivating property of serum was [illegible]
precipitated [illegible]
40 minutes [illegible]
phages acc[illegible]
in deflection in the inactivation curve was observed compared to serum controls handled in the same way but not treated with CO The experiment was repeated with the same and with another serum with consistent results The exposure to CO_2 during the storage could not be very intense as the solid CO was kept outside a metal box containing the sera

Reference serum stored at — 60° C was tested daily according to the standard procedure during an investigation on the variation of inactivating capacity of normal sera drawn from persons of different physiological and pathological state (to be published) Six reference sera observed for periods varying from 2 to 6 months were analysed with respect to the serum concentration necessary to inactivate 50 per cent of the phages, the slope of the linear part of the inactivation curve and the level of the persistent fraction No significant difference was observed in these respects with increasing time

Eighteen ordinary serum specimens stored at — 60° C were retested after periods varying from 3-16 months The serum concentrations able to inactivate 50 per cent of the phages were not significantly increased The slope of the curves was calculated according to the equation

$$\frac{\log p}{p_0} = k \log c \quad a$$

in which p_0 = the initial number of phage particles, p = the number of phage particles remaining active c = serum concentration, k = a

4° C is shown in Fig 3 The slope of the straight part of the curve does not seem to decrease with the time of storage as at 37° C On the other hand the gradual loss of inactivating effect and the increase of the persistent fraction was the same as seen at 37° C

TABLE 3

Decrease of Phage Inactivating Capacity after Incubation of two Sera at 22° C

Time	Serum			
	I		II	
	Reciprocal of serum dil inactivating 50% of the phages	Serum activity in %	Reciprocal of serum dil inactivating 50% of the phages	Serum activity in %
–	30 8	100 0	40 1	100 0
12 hours	29 3	95 1		
18 „	30 9	100 3		
24	28 1	91 2		
30 „	29 4	95 5		
2 days	25 1	81 5	27 9	69 6
3 „	24 4	79 2	26 4	65 8
4	17 8	57 8	26 0	64 8
5			15 9	39 7
6			10 2	25 4
7			6 2	15 5
8				

Reaction mixture Total volume 1 ml about 1×10^{8} T 2 particles phage and serum (I A 6, II A I A) dil in barbiturate buffer 0 125 M at pH 7 4 + 0 05 0 0025 M Mg Incubated at 37° C for 2 hours

TABLE 4

Decrease of Phage Inactivating Capacity after Storage of two Sera at 4° C

Days	Serum			
	I		II	
	Reciprocal of serum dil inactivating 50% of the phages	Serum activity in %	Reciprocal of serum dil inactivating 50% of the phages	Serum activity in %
	40 1	100 0	38 5	100 0
1			41 2	107 0
2	38 5	96 0	39 2	101 8
4	35 8	89 3	38 0	98 7
6	24 3	60 6		
8	16 7	41 6		
10	11 1	27 7		
12	8 5	21 2	25 8	67 0
14			19 8	51 4
18			17 5	45 5
24				

Reaction mixture Total volume 1 ml about 1×10^{8} T 2 particles phage and serum (I A I A II A A) dil in barbiturate buffer 0 125 M at pH 7 4 + 0 05 0 0025 M Mg Incubated at 37° C for 2 hours *Duplicate experiments*

uspension + nutrient broth 238 determinations of the phage activity after incubation for one or two hours at 37° C with heated serum were compared with the corresponding determinations (226) in broth controls 119 of 166 heated sera proved to retain some activity The mean phage activity in all serum controls was found to be 93 4 per cent of the mean of the broth controls No decrease in the relative number of survivors was observed after a reaction time of two hours in comparison to one hour In a triplicate experiment four different sera heated at 56° C for 30 minutes were diluted 1 5 and incubated with phages for 24 hours at 37° C In comparison to the plaque counts of broth controls treated in the same way 87 7 per cent of the phages were infectious Thus the progress of inhibition with time if any must be very slow

The stability to temperatures between 45° and 70° C was examined in a series of experiments An ultra thermostat waterbath accurate for ± 0 02° C was used The temperature was determined with the aid of a calibrated thermometer with a precision of ± 0 02° C Firstly the individual thermoresistance of seven different sera was determined by heating undiluted sera for one hour at temperatures from 45° to 69 C The sera were then diluted in barbiturate buffer and mixed with phage suspension to a final serum dilution of 1 10 Six sera were found to behave in about the same manner In several duplicate experiments the activity was critically destroyed between 47° and 49° C When incubated with sera heated at 49° 53° C the number of active phage particles was a few per cent higher than in the broth controls (4 5 broth controls in each experiment) The number of infectious phage particles decreased when the sera were exposed to temperatures above 56° C Maximum inhibiting effect on an average 29 1 per cent of the controls was reached after heating the sera at 62° C The activity of the seventh serum was destroyed more gradually within the temperature range 48° 52° C The inactivating effect was not completely abolished the inactivated virus fraction never being less than 15 per cent

Two (A and B) of the six sera showing a similar thermoresistance and the serum with aberrant properties (C) were selected for further examination of the stability at 45° 70° C for one hour The inactivating capacity of the heated sera was tested against a comparatively fresh phage lysate (4 weeks) and an old one (7 months) The means of all fresh and old phage determinations at each temperature tested are listed in Table 7 The loss of serum activity at a critical temperature and the appearence of phage inhibition above this temperature as observed in the preliminary experiments was confirmed The virus inhibiting effect of serum especially marked after heating at 62° C seemed gradually to [illegible]

phage [illegible]
contro[illegible]
fresh [illegible] per cent

constant indicating the slope and a = a parameter (*Kallings* 1961 a). Table 5 lists the quotient between the k values of fresh and stored serum. The *differences* between the persistent phage fractions with fresh and stored serum represented by the per cent phage activity remaining at the serum dilution 1 : 7.5 are also shown in this table. The slope of the curves was found to decline with the time of storage while the persistent fraction was increased, showing thus in some respects the same type of phenomenon as appears after storage of serum at higher temperatures.

TABLE 5

Modification of Phage Inactivating Capacity after Storage at —60° C

Months	Ratio between k values of fresh and stored serum	Levels of persistent fraction of stored serum in relation to fresh serum	Number of sera tested
6	1.0	+ 2.0 %	3
7–12	1.3	— 8.1 %	13
13–16	1.7	—13.8 %	5

It may be mentioned in this connection that repeated freezing and thawing of fresh sera was not found to influence the virus inactivating potency.

The observations pertaining to serum stability at 37° C and below are summarized in Table 6.

TABLE 6

Stability of the Phage Inactivating Capacity of Undiluted Normal Serum at —60° C to +37° C

Temperature	Stability	50 per cent of initial activity
—60° C	6–12 months	
—30° C	2 months	
+ 4° C	1–4 days	6–18 days
+22° C	24–30 hours	4–5 days
+37° C	8–12 hours	2–3 days

Stability above 37° C

Investigation of the stability above 37° C was induced by two interesting observations. Firstly, the inactivating system of normal serum was found to resist heating at 45° C for at least two hours entirely (*Kallings* 1961 b). Secondly, it was observed that the activity was not completely destroyed by heating at 56° C for 30 minutes or that there was an unexpectedly high number of sera containing heat-stable inhibitors.

In a large number of experiments two kinds of controls were used to determine the initial phage activity, namely equal portions of phage suspension + undiluted serum heated at 56° C for 30 minutes or phage

mostability of active serum factors, the sera were also heated after dilution 1 5 in barbiturate buffer at temperatures between 47° and 62° C and tested for remaining activity The loss of activity in these serum dilutions was the same as in undiluted sera

Finally, the effect of heating at 80° C for one hour was tested Owing to coagulation it was necessary to dilute the three sera 1 10 prior to the examination After heating at this temperature no virus inhibiting activity remained

Kinetics of the phage inhibiting action of heated sera In a duplicate experiment, sera A and B were first incubated at 62° C for one hour, then serially diluted, mixed with phages and incubated for varying periods at 37° C to examine the time and concentration dependence of the reaction between phages and heated serum The sera were diluted 1 10, 1 20, 1 40 and 1 80 and incubated with phages for 1, 2 and 4 hours The number of active phages did not change with increasing reaction time The mean phage activity at each serum dilution (12 observations) was 86 4 (1 10), 85 0 (1 20), 86 8 (1 40) and 90 0 (1 80) per cent of the mean of 4 broth controls Thus there seems to be no relationship between the decrease in active phages and the concentration of serum within the range tested It was experienced in previous experiments that the number of infectious phage particles in mixtures of phage and low concentration of native serum might decrease below the number obtained in broth controls when serum was diluted more than 1 50 1 100 and when the reaction mixtures were incubated at 37° C for some hours This decrease may be due to a "spontaneous" loss of infectivity due to the lessened amount of protective colloids and (or) the appearance of a phage inhibiting effect The percentage of phage activity obtained at serum dilution 1 80 may thus be uncertain

The difference in thermostability appearing between serum C and the other sera might be due to different concentrations of factors necessary for the inactivation of phages The original phage inactivating capacity of the 7 sera used was therefore determined according to the standard inactivation test The strength of the sera expressed as the reciprocal of the dilution inactivating 50 per cent of the phages is shown in Table 8 In [illegible] ion of duplicate in act[illegible] 8 1 per cent of the me[illegible] stronger than the oth[illegible]

The original [illegible] 50° 45 50° and 54° C were tested)[1] The comparison between unheated sera showed that the complement activity of serum C was significantly ($p < 0.001$) higher than that of serum B though the difference was very small (Table 9)

Heating was found to diminish the hemolytic power of the two sera at the same rate As much as 43 46 per cent activity remained after heating for one hour at

[1] Thanks are due to Dr *B Heferstedt* for kind help and advice

TABLE 7

Decrease in Phage Inactivating Capacity of Serum at 37°–70° C for 1 Hour
Mean Per Cent Phage Activity after Incubation with Heated Serum Diluted 1 10 for 2 Hours at 37° C

Serum heated at °C	Lysate Serum A I	Lysate Serum A II	Lysate Serum B I	Lysate Serum B II	Mean A+B I+II	Total nr of observations	Lysate Serum C I	Lysate Serum C II	Mean	Total nr of observations
37 10	17 1		11 8		14 5	6	3 4		3 4	6
45 13	19 9		14 1	10 7	16 2	12	6 6	7 3	7 0	8
47 13	23 1		28 6		24 6	16	4 3		4 3	6
47 63		84 1		85 0	84 6	4		9 8	9 8	2
48 13	88 4	109 9	84 4	105 0	92 7	14	23 2	22 2	22 9	6
49 14	117 0		102 5	101 8	109 6	10	37 9		37 9	2
50 14	101 1	103 2	99 4	103 2	102 0	10		53 4	53 4	2
51 14	110 5	92 4	98 8		101 7	10	65 7		65 7	2
52 14	93 0	91 7	93 6	93 3	93 0	18		78 4	78 4	4
53 14			93 2		93 2	2	85 5		85 5	2
54 14	96 5	97 3	80 6	94 0	92 1	16	71 0	87 2	81 9	6
56 14	89 1	95 3	87 2	114 0	92 2	32	76 4	89 0	80 2	12
58 14	82 1	95 0	101 6	93 2	93 0	16				
60 14	77 7	82 5	87 1	99 1	85 8	20	69 0	87 0	78 0	8
62 13	70 3	80 0	81 2	81 9	77 8	22	49 1	72 5	64 7	6
64 12	88 4	82 0	87 4	115 5	93 4	8	80 6	96 8	88 7	4
66 11	89 6	90 2	101 9	105 9	96 5	12	90 1	76 2	83 2	4
70 10	96 0		96 0		96 0	5	95 5		95 5	2

T 2 lysate I 7 months II 1 month old

Reaction mixtures Total volume 1 ml 0 1 ml heated serum + 0 4 barbiturate buffer 0 125 M pH 7 4 ± 0 05 + about 1 5 × 10[4] T 2 particles in 0 5 ml barbiturate buffer 0 0025 M Mg Controls 0 5 ml nutrient broth + 0 5 ml T 2 suspension as above

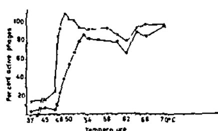


Fig 4

Decrease in phage inactivating capacity of serum heated at 37 70° C according to Table 7

Means of all observations with sera A & B (left curve) and with serum C (right curve)

The means of all determinations of the phage activity after incubation with sera A and B on the one hand and with C on the other are graphically presented in Fig 4 The number of observations at each temperature is listed in Table 7 To avoid coagulation the sera were diluted 1 2 in barbiturate buffer before heating at 62 C and 1 5 before heating at 64°, 66° and 70° C As dilution might change the ther-

mostability of active serum factors, the sera were also heated after dilution 1 5 in barbiturate buffer at temperatures between 47° and 62° C and tested for remaining activity The loss of activity in these serum dilutions was the same as in undiluted sera

Finally, the effect of heating at 80° C for one hour was tested Owing to coagulation it was necessary to dilute the three sera 1 10 prior to the examination After heating at this temperature no virus inhibiting activity remained

Kinetics of the phage inhibiting action of heated sera In a duplicate experiment, sera A and B were first incubated at 62° C for one hour, then serially diluted, mixed with phages and incubated for varying periods at 37° C to examine the time and concentration dependence of the reaction between phages and heated serum The sera were diluted 1 10, 1 20, 1 40 and 1 80 and incubated with phages for 1, 2 and 4 hours The number of active phages did not change with increasing reaction time The mean phage activity at each serum dilution (12 observations) was 86 4 (1 10), 85 0 (1 20), 86 8 (1 40) and 90 0 (1 80) per cent of the mean of 4 broth controls Thus there seems to be no relationship between the decrease in active phages and the concentration of serum within the range tested It was experienced in previous experiments that the number of infectious phage particles in mixtures of phage and low concentration of native serum might decrease below the number obtained in broth controls when serum was diluted more than 1 50 1 100 and when the reaction mixtures were incubated at 37° C for some hours This decrease may be due to a "spontaneous" loss of infectivity due to the lessened amount of protective colloids and (or) the appearance of a phage inhibiting effect The percentage of phage activity obtained at serum dilution 1 80 may thus be uncertain

The difference in thermostability appearing between serum C and the other sera might be due to different concentrations of factors necessary for the inactivation of phages The original phage inactivating capacity of the 7 sera used was therefore determined according to the standard inactivation test The strength of the sera expressed as the reciprocal of the dilution inactivating 50 per cent of the phages is shown in Table 8 In previous experiments the standard deviation of duplicate inactivation tests performed on different days was found to be ± 8 1 per cent of the mean titer (*Kallings* to be published) Serum C proved to be str[illegible] thers th[illegible]

[illegible] at 46 48° 50° and 54° C were tested[1] The comparison between unheated sera showed that the complement activity of serum C was significantly ($p < 0.001$) higher than that of serum B though the difference was very small (Table 9)

Heating was found to diminish the hemolytic power of the two sera at the same rate As much as 43 46 per cent activity remained after heating for one hour at

[1] Thanks are due to Dr *B Hederstedt* for kind help and advice

48° C As was expected no hemolytic activity was revealed after heating at 50° or 54° C for one hour with the test system used nor when a surplus of serum was tested

TABLE 8

Original Phage Inactivating Capacity of 7 Normal Sera Used for the Investigation of the Stability at 37°–70° C

Serum	$\bar{x}$ Reciprocal of serum dilutions inactivating 50 per cent of the phages
A	38 5
B	25 0
C	54 1*
D	35 1
E	46 5*
F	45 9
G	43 1

Duplicate experiment
* Means of repeated tests
Reaction mixture Total volume 1 ml phage and serum diluted in barbiturate buffer, 0 125 M pH 7 4 ± 0 05 about 1 × 10¹ T 2 particles 0 0025 M Mg⁺⁺ Incubated for 2 hours at 37° C

TABLE 9

Decrease in Hemolytic Activity of Serum Heated at 46° 48° and 50° C for one Hour

Serum	Serum			
	B		C	
Sample	Activity in 50 per cent hemolytic units	Per cent remaining activity	Activity in 50 per cent hemolytic units	Per cent remaining activity
Unheated	121 3	100	133 0	100
46° C	103 9	85 7	114 8	86 3
48° C	52 6	43 4	61 0	45 9
50° C	<5 0	<4 1	<5 0	<3 8

Reaction mixture Total volume 1 25 ml 0 75 ml serum dilution in veronal—NaCl buffer 0 5 ml sensitized sheep erythrocytes Incubated in a waterbath at 37° C for 45 minutes Hemolysis estimated spectrophotometrically 50 per cent units calculated according to von Krogh s equation

Thus the dissimilar behaviour of heated serum C in the phage inactivation test does not seem to be explained neither by definitely higher titers of phage inactivating power or complement activity nor by an increased stability of the thermolabile complement factors (C 1 and C 2)

DISCUSSION

The inactivation of phages by normal serum is known to be a complex reaction in which several serum factors participate *Van Vunakis et al* 1956 and *Barlow et al* 1958 presented evidence for the requirement of properdin and C'1 4 The properdin requirement was also demonstrated by *Wedgwood et al* (1956), *Landy et al* (1958, and *Hinz*

et al (1960) On the other hand several authors have reported results indicating that the properdin system alone is not essential for the phage inactivation (*Cowan* 1958 *Nelson* 1958 *Pernis & Turri* 1958 *Toussaint & Muschel* 1959) According to these the phage neutralizing potency of fresh serum is (also) thought to be dependent on the presence of normal antibodies A discussion of the diverse findings does not fall within the scope of the present paper they are only mentioned to give an outline of the factors which may participate in the reaction Several other factors have to be considered in addition to those related for example the principle in serum activating virus particles inhibited by host products (*Kallings* 1961 a) and the occurrence of inhibitors to the neutralization process (*Kallings* 1961 b d)

Most factors defined and undefined are likely to be inhibited or denatured at different temperatures and to form inactive or partially active complexes with other serum components varying with the temperature Thus it is evident that heating of serum will have a very complex effect on the phage inactivating ability as revealed by the present findings (Fig 4)

The hemolytic activity of guinea pig C 1 and C 2 is lost by heating for 30 minutes at 50° C (*Pillemer et al* 1941) C 3 and C 4 are inactivated at 63° and 66° C respectively (*Ecker & Pillemer* 1942 *Pillemer et al* 1941) Storage in an ice box for 3–4 days may reduce the C activity by 90 per cent (*Boyd* 1956) *Pillemer et al* (1942) studying the complement activity in specific immune fixation found that fresh guinea pig serum lost 50 per cent of its complementary activity during 19 hours in the cold The complement is known to be stable for prolonged storage in sealed ampoules frozen on solid CO (*Kabat & Mayer* 1948)

Pillemer et al (1954) found properdin in serum to be stable at 18° C partly inactivated at 50° C and completely inactivated at 56° C for 30 minutes It was stable for 1 2 days at + 1° C and for a long time at 70 C Purified properdin is stable at 66° C for 30 minutes but is inactivated at 100° C for 5 minutes and after two months at + 1° C (*Pillemer* 1956)

For comparison conventional immune antibodies are known to be [illegible]

[illegible] minutes to reduce the specific neutralizing capacity *only slightly* while heating at 80 C almost abolished it Heating at 75° C destroyed their ability to cause precipitation but not to fix the complement However *Heidelberger et al* (1946) observed that heating weak human antipneumococcus sera at 56° C for 30 minutes reduced the amount of precipitable antibody markedly

Within the temperature range 0° to 37 C the phage inactivating potency of normal serum as found in the present study seems to be more stable than the hemolytic activity of the complement and showed

about the same stability as the activity of properdin measured by the zymosan method

As reported above, the virus inactivating capacity of diluted serum was more thermolabile than that of undiluted serum An analogous phenomenon has been observed for the thermal inactivation of the complement *Cavallo* (1958) found that the deterioration of diluted guinea pig complement during 45 minutes at 37° C was about 15 per cent while it was almost negligible when undiluted sera were treated in the same way The phage neutralizing capacity was, however, stable during 4 hours at 37° C Thus thermal inactivation of the phage neutralizing system would not occur during the reaction times ordinarily used in investigations of this activity

Landy et al (1958) reported that heating pools of fresh human sera at 37° C for one hour considerably increased the T2 phage neutralizing action Only some individual sera were influenced in this way In the present experiments individual sera were used throughout A tendency towards steeper slopes of the inactivation curves was observed after incubation of some sera at temperatures between 37° C and 45° C, the increase was, however, within the error of the method

The decreased slope of the inactivation–serum concentration curve which appeared during storage of serum for more than 6 months in the CO_2 ice box may be due to the formation or release of inhibitors to the virus neutralizing activity The sensitivity of the neutralizing reaction to the action of inhibitors initially present or ordinarily formed during the progress of the reaction may also be increased by the deterioration of substances participating in the virus inactivation Modifications of the factors participating in the neutralization reaction, leading only to quantitative variations in the total neutralizing potency should displace the curve without altering the slope

The analysis of time phage survivor curves of sera pre-incubated at 22° C or 37° C revealed that the most marked effect was not only a slowing of the constant inactivation rate but a prolongation of the initial lag period and an earlier deviation from the linear to the retarded rate of reaction, especially evident at the higher serum concentrations The phage activating effect, evident at low serum concentrations, was not found to decrease while the neutralizing power was partly inactivated These circumstances would contribute to the flattening of the serum concentration inactivation curves observed

What causes the neutralization reaction deflect from the linear course at an earlier level is not clear although an inhibitor effect might be a plausible explanation for the phenomenon observed when serum is kept at temperatures between — 60° and + 45° C

The fact that incubation of phages with high concentrations of normal serum previously heated at 49° 53° C raised the number of infectious particles in comparison to the number obtained in broth controls points to a phage activation effect previously only demonstrated at low

serum concentrations (*Kallings* 1961 a) However the discontinuous course of the time survival curve at the reaction temperature 45° C and the serum dilution 1 5 previously reported (*Kallings* 1961 b) and the present findings indicate that the activating capacity of normal serum is not only a property of low concentrations of fresh sera The activating effect seems to occur also in high serum concentrations but is generally masked by the high phage inactivating power The activating principle seems to resist heating to 56° 66° C for one hour as revealed by the experiments summarized in Table 7

Contrary to previous findings (*Van Vunakis et al* 1956 *Landy et al* 1958) heating at 56° C for 30 minutes was not found to destroy the phage inhibiting ability of normal serum completely The remaining activity was not limited to an insignificant number of sera but was a common property demonstrated in 72 per cent of the tested sera

It is worthy of note that the phage inhibiting effect seemed to be nullified at 70° 80° C temperatures known to destroy the activity of immune antibodies There appears to be no reason however to classify the phage inhibiting activity observed at and above 56° C as due to conventional antibodies or heat stable inhibitors originally present in an active state in the serum as the serum effect is abolished at about 80° C Considering this and the changed kinetics of the inhibition it is thought to be due to non specific inhibiting substances formed or released during the heating of most sera

SUMMARY

The phage inactivating capacity of normal serum is found to be stable during two hours at 45° C and to be destroyed during one hour at 48 C When serum is heated at temperatures above 52° 53° C a limited virus inhibiting effect appears in the majority of sera This effect is most marked when serum is heated at 62° C and disappears between 70 and 80 C

Serum activity is stable during about 8 12 hours at 37° C about 24 30 hours at 22° C 1 4 days at + 4° C 1 2 months at — 30° C and 6 12 months at 60 C The deterioration of the neutralizing ability increases when serum is diluted prior to incubation

REFERENCES

1 *Bart J I Van Vunakis Helen & Levine L* Stul [illegible]

2 *B xl* [illegible]

3 *I pd* [illegible] mun l gy Interscience Publishers N Y p 357 1956

4 *Cann J R & Clark E W* On the kinetics of neutralization of bacteriophage T 2 by specific antiserum J Immun l 72 463 477 19 4

5 *Cavallo V* Dilution effect on thermal inactivation of complement VIIth Internat Congr Microbiol Stockholm 1958 Abstracts 10 g p 171

6 *Cowan K M* Interpretation of properdin levels determined by phage neutralization technique Science *128* 778 79 1958

7 *Ecker E E & Pillemer L* Complement Ann N Y Acad Sci *43* 63 83 1942

8 *Hedersted B* Studies on the complement activity in the Treponema pallidum immobilization (TPI) test A comparative study of the immobilizing and hemolytic complement activity Acta path et microbiol scandinav (to be published)

9 *Heidelberger M Mac Leod C M Kaiser S J & Robinson Betty* Antibody formation in volunteers following injection of pneumococci or their type specific polysaccharides J Exp Med *83* 303 319 1946

10 *Hin- C F Wedgwood R J Todd E W & Pillemer L* The properdin system and immunity XIV The injection of human properdin in rabbits and the production of antibodies to properdin J Immunol *85* 547 558 1960

11 *Kabat E A & Mayer M M* Experimental Immunochemistry C C. Thomas Springfield Ill p 112 1948

12 *Kallings L O* Studies on the inactivation of bacterial viruses by normal human serum

I Kinetics of the coli T 2 phage inactivation at various serum concentrations Acta path et microbiol scandinav *51* 389 402 1961 a

II Effect of phage concentration temperature pH and molarity on the kinetics of the coli T 2 phage inactivation Acta path et microbiol scandinav *52* 71 81 1961 b

III Reactivation of inactivated coli T 2 phages Acta path et microbiol scandinav *52* 82 97, 1961 c

V The influence of heated and zymosan adsorbed serum on the inactivation of coli T 2 phages Acta path et microbiol scandinav (in press 1961 d)

13 *Landy M Trapani R J & Shear M J* Interference of endotoxic polysaccharides with inactivation of coliphage by human serum Brit J Exp Pathol *39* 618 630 1958

14 *Nelson R A* An alternative mechanism for the properdin system J Exp Med *108* 515 535 1958

15 *Pillemer L Seifter S &* complement in specific immune

16 *Pillemer L Ecker E E* on and physicochemical characterization of the serum protein components of complement J Exper Med *74* 297 308 1941

17 *Pillemer L Blum Louis Lepow I H Ross O A Todd E W & Wardlaw A C* The properdin system and immunity I Demonstration and isolation of a new serum protein properdin and its role in immune phenomena Science *120* 279 285 1954

18 *Pillemer L* The nature of the properdin system and its interactions with polysaccharide complexes Ann N Y Acad Sci *66* 233 243 1956

19 *Pernis B & Turri M* Osservazioni sul dosaggio della properdina mediante la neutralizzazione del batteriofago Boll Ist Sieroter Milan *37* 414 424 1958

20 *Toussaint A J & Muschel L H* Neutralization of bacteriophage by normal serum Nature *183* 1825 1827 1959

21 *Vanakis H Van Barlow J L & Levine L* Neutralization of bacteriophage by the properdin system Proc Nat Acad Sci *42* 391 394 1956

22 *Wedgwood R J Ginsberg H S & Pillemer L* The properdin system and immunity VI The inactivation of Newcastle disease virus by the properdin system J Exp Med *104* 707 725 1956

MORPHOLOGICAL EVIDENCE OF HEMATOGENOUS DISSEMINATION OF PARAGONIMUS WESTERMANI

By

JØRGEN RINGSTED and KI HONG KIM

Received 12 viii 60

It has not been settled with certainty whether ectopic infection by the lung fluke (Paragonimus Westermani) is brought about by hematogenous dissemination or by migration of the parasites through soft tissues

Chu 1956 in a review of the litterature concerning ectopic infection by the lung fluke favoured the latter hypohesis which is mainly based on experimental studies in dogs (*Kawamura & Matsui* 1916 *Ando* 1917 and *Yokowa & Suyemori* 1919 and 1921) The latter authors found that (1) Immature parasites did not migrate into the cranial cavity of dogs when introduced in the sides of their necks (2) Young distomas did not penetrate into the cranial cavity when introduced into the orbits of dogs but in some cases made their way into the pleural cavity 3) Young distomas introduced into the common carotid of dogs were not being carried into the cranial cavity but in several cases were encountered in the pleural cavity and (4) When both larvae and adults were introduced directly into the cranial cavity of dogs only one out of over one hundred worms were later on found in the cranial cavity but a number were met with in the lungs or the pleural cavity The authors suggested that the parasites had left the cranial cavity by the venous sinuses and the cranial cavity to be an unfavourable location for the development of Paragonimus Westermani This conclusion is well founded but on the other hand the experiments failed in demonstrating the routes used by the parasites towards *the brain* The experiments do not speak in favour of hematogenous dissemination but neither do they support the previously forwarded opinion that the high incidence of cerebral paragonimiasis in children might be due in [illegible]

[illegible]

Other authors quoted from (*Chu*) have held the opinion that the flukes are spread by the blood stream Thus *Musgrave* 1907 found lesions in every organ except the stomach presumably due to hematogenous dissemination *Kim* 195[illegible] observed that in cerebral para



Fig 1
Aorta with multiple minute depressions of the intima

gonimiasis the left side of the brain was more frequently involved than the right side, and related this observation to the fact that the left carotid artery is a direct branch of aorta which is not the case with the right carotid artery However, the number of observed cases seems too small to allow any conclusions

At the National Medical Center in Korea we have observed a case of paragonimiasis with changes of aorta, pulmonary artery, and atria of the heart which render it highly probable that the parasites have penetrated the walls of these structures, the observations thus strongly suggest that Paragonimus Westermani at least is able to spread by the blood route

CASE REPORT

The patient was a 7 year old boy who was admitted to the Pediatric Department NMC in May 1959. His main complaints were cough with dyspnoea and severe general weakness he had hemoptysis once He was in a very poor condition emaciated

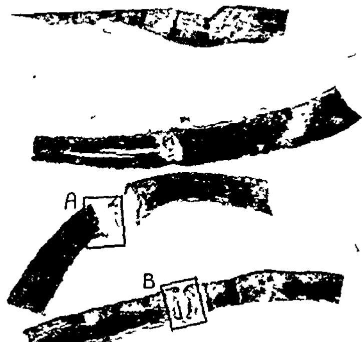


Fig 2
Verhoeff stained sections of aorta showing canals through the whole wall with more or less complete loss of elastic membranes and replacement by simple connective tissue scars

anemic, and dyspnoeic he had generalized edema and was running an irregular fever There were no neurological symptoms Chest X ray revealed extensive bilateral densities Mantoux was negative and sputum contained no Tb but eggs of Paragonimus Westermani were demonstrated in the sputum

Autopsy (A 117 59) showed paragonimiasis with massive involvement of lungs, liver, pleura, pericardium, peritoneum, and probably brain Additional findings were adhesive pericarditis, bilateral pleuritis, and peritonitis The peritoneal cavity contained small sacks containing yellowish brown material, in the latter numerous eggs were found and Salmonella paratyphosa B was grown on cultivation Numerous adult worms were found in characteristic cavities in the lungs and in the liver both on macroscopical and microscopical examination Tremendous numbers of eggs were demonstrated in the organs mentioned, especially in the peritoneal cavity The right cerebral hemisphere exhibited a 5 × 5 × 5 cm hemorrhage in front of the central sulcus, but neither worms nor eggs could be demonstrated in sections from this lesion No other parasitic diseases were encountered at the autopsy

In the ascending aorta and in the aortic arch about 15 minute, round depressions of the intima were found, it looked as if this limited area of the aortic intima had been pricked repeatedly with a fine needle



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Aorta with multiple minute depressions of the intima

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Fig 4
Larger magnification of Fig 2 (B)

adults of Paragonimus Westermani are able to migrate through dense membranes as the aorta, and (2) In this patient larvae or young adult worms have at some time been present in considerable number in the lumen of the vascular system

The aortic lesions were scattered over a rather limited area of the intima, this makes it most probable that the parasites have made their way through the vessel wall from the outside towards the lumen of the aorta, had the opposite route been followed, one would expect that the "tracks" of the parasites had been spread over a larger area of the intima Besides, it seems unlikely that the parasites should have been able to attach themselves sufficiently to the intima of aorta to omit being washed away by the blood stream Whatever the migration route through the aorta has been the findings indicate that (3) the migration of the Paragonimus Westermani is *not* necessarily directed toward the tissues offering "less impediment to the worms' migration", as previously supposed



Fig 3
Larger magnification of Fig 2 (A)

(Fig 1) *Microscopic examination of serial sections of these peculiar* aortic lesions showed that they represented about 1 mm thick punched out canals right through the wall of the aorta, so that the normal structures of the wall were completely replaced by a more or less cellular, occasionally vascularized connective tissue in some cases with the *character of small aneurysms (Figs 2, 3, and 4) Similar lesions were* found in both atria of the heart and in the pulmonary artery, and finally microscopic examination showed a similar scar in a small vessel in pancreas Hemosiderophages were seen in adventitia corresponding to the lesions

DISCUSSION

The *observed* vascular lesions *in a well established* case of widely disseminated paragonimiasis do not match any specific vascular disease, they can hardly have been produced by anything but *the migration* of parasites through the vessel walls

The findings thus make it highly probable that (1) Larvae or young

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PRESENT STATUS OF LABORATORY STUDIES ON TOBACCO CARCINOGENESIS

By

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Received 5.ix.60

Definitive proof that smoking is a cause of cancer in man rests upon epidemiological studies. Statistical surveys, both of the retrospective and prospective type, have shown without exception that smoking, particularly of cigarettes, has a high association with cancer of the lung. In the opinion of several public health authorities the association is regarded as causative (1, 3, 7, 9, 10, 18, 20, 23).

The basic significance of laboratory studies does not lie in adding to the proof that smoking is a cause of cancer in man, but rather in defining the various steps involved in tobacco carcinogenesis and in permitting studies leading to safer smoking products. It is along these lines that our research program is directed. The present report concerns the current status of laboratory research in this field.

REDUCTION IN SMOKE CONDENSATE

Statistical studies have shown that the risk of developing lung cancer increases with the amount of tobacco consumed (4, 5, 8). Similarly, biological experiments have demonstrated that the number of tumors obtained in mice is related to the amount of tobacco smoke condensate applied (26). For these reasons studies were undertaken for possible ways of reducing the amount of smoke condensate of cigarettes.

Filter Cigarettes. A previous publication showed major differences in smoke condensate in different types of American cigarettes (25). Among the ten leading American cigarettes the amount of smoke condensate ranged from 30.8 mg to 17.7 mg per cigarette. In general, king size cigarettes (85 mm) were found to be higher in smoke condensate and nicotine content than regular cigarettes (70 mm) when smoked under standard conditions to the same butt length (23 mm) and cigarettes with filter tips were found to be lower than plain cigarettes. A reduction in smoke condensate can also be achieved through tobacco selection and high porosity or perforated paper.

These studies indicated that the presently used filter materials do not selectively remove carcinogens or promoting substances from to-

It is tempting to relate the factors that govern the migration of the worms to high oxygen tension. This would explain that flukes prefer the lungs, and also maybe that the brain is "an unfavourable location for the development of Paragonimus Westermani", once located in the brain the worms will cause infarction and thus themselves abolish their possibilities of oxygen supply. They probably then die and are resorbed, or migrate to more favourable locations after having deposited some eggs, that the latter event actually may occur was demonstrated by the above mentioned experiments of *Yokogawa & Sugemori*, where a hematogenous route was considered most likely.

SUMMARY

In the aorta, the pulmonary artery, and both atria of a 7 year old boy who died from disseminated paragonimiasis small scars were found at autopsy. The nature of the scars indicates that they were tracks of parasites which had penetrated the vessel walls. The observation lends strong support to the concept that Paragonimus Westermani is able to spread hematogenously.

REFERENCES

Chu H. Ectopic Infection by the Lung Fluke, Nat. Inst. Prev. of Infect. Dis. Min. Health & Soc. Aff. Republic of Korea Nov. 1956.

Kim S. K. Cerebral paragonimiasis report of four cases. J. Neurosurg. *12* 89-94 1955. (Quoted from *Chu*)

Musgrave W. F. Paragonimiasis in Philippine Islands, Philippine J. Sc. *B2* 15-63 1907. (Quoted from *Chu*)

Yokogawa S. & Sugemori S. An experimental Study of the Intracranial Parasitism of the Human Lung Fluke Paragonimus Westermani. Am. J. Hyg. *1* 63-78 1921.

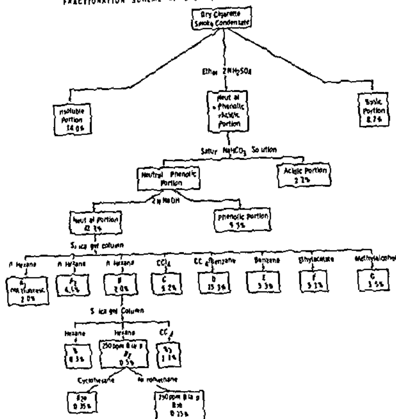


Fig 1

Additives In an attempt to reduce the amount of polynuclear hydrocarbons we added a number of chemicals to tobacco before making them into cigarettes In each instance the material was added in powder form to standard cigarette tobacco in a concentration of 4 to 5 per cent The selection of the additives is based mainly on the following points

1 Possible change of combustion temperature and conditions cobalt (III) oxide and calcium carbonate

2 The interaction with the thermic formed radicals of aromatic hydrocarbons copper (II) nitrate or with aromatic hydrocarbons themselves cholic acid as it occurs under experimental conditions

3 The use of some oxides and silicates is employed in various catalytic processes aluminum oxide trihydrate and anhydrous (activated and non activated)) aluminum silicate magnesium oxide and boric acid

bacco smoke condensate However, in view of the fact that most filter cigarrettes tend to reduce the total smoke condensate, provided a smoker does not significantly increase his smoking habits, cigarettes with effective filters represent a safer smoking product than plain cigarettes

Butt Length Studies determining the smoke condensate and nicotine content of the first and second half of *cigarettes showed that the* second half of the cigarette contains 41 to 45 per cent more smoke condensate than the first half and the nicotine amount rises similarly (25)

Puff Frequency Studying the puff frequency per cigarette indicated that the amount of smoke condensate markedly increases with the frequency of puffs per cigarette (25) Smoking one of the 85 mm cigarettes, once a minute, gave a reading of 35 1 mg of smoke condensate, twice a minute—53 0 mg, and three times a minute—64 1 mg

Inhalation Studying the extent of inhalation by measuring the variations of inhaled and not inhaled aerosols of cigarette smoke showed that if the smoker takes the smoke only into his mouth for approximately two seconds, about 10 per cent of the aerosols are retained On the other hand, a smoker who deeply inhales will retain up to 90 per cent of the smoke aerosols (25)

In summary, clinical laboratory studies indicate that safer smoking would include the use of a type of cigarette, which is as low in smoke condensate and nicotine content as is commensurate with smoking satisfaction, which essentially means an effective filter cigarette They also suggest that cigarettes should not be puffed too frequently or smoked to the butt and that the smoke should not be deeply inhaled

THE IMPORTANCE OF POLYCYCLIC HYDROCARBONS

On the basis of previously published biological and chemical studies it has been proposed that the identified carcinogenic polycyclic hydrocarbons represent a major group of initiating carcinogens in tobacco *smoke condensate* (17, 24) The *polynuclear aromatic* hydrocarbons are mainly formed during the combustion of the tobacco The tobacco of our standard cigarettes contains only very minute quantities of benzo(a)pyrene (0 02 ppm) A bioassay indicates that these polycyclic hydrocarbons *of the condensate by themselves, however, can account* for not more than 3 per cent of the total biological activity and on the basis of the most potent single fraction from the condensate, in which the aromatic hydrocarbons are enriched more than 50 times (Fraction B, Fig 1), *these compounds can account for only 10 per cent of* the established biological activity It has, therefore, been clear that additional carcinogens or co-carcinogens have to be present Fig 1 shows the procedures currently in use for chemical separation of cigarette smoke *condensate*

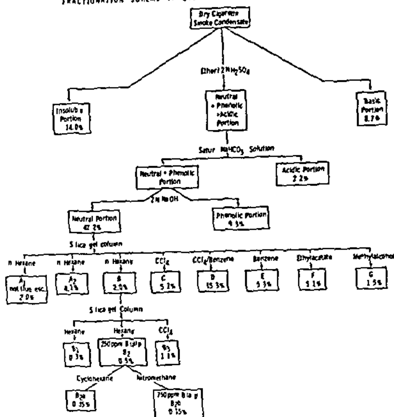


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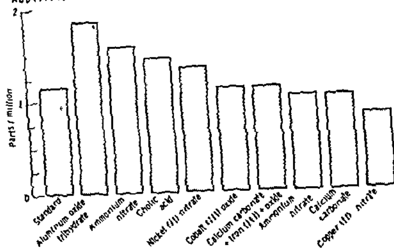


Fig 3

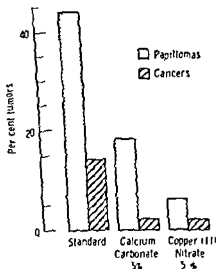


Fig 4

sium oxide and aluminum silicate Chemical determinations for polycyclic hydrocarbons *in these condensates*, carried out on the condensate smoked once a minute, show an *increase in benzo(a)pyrene* for several condensates especially for the *aluminum* oxide trihydrate (1 8 parts per million) and a decrease for *copper nitrate* (0 8 parts per million) compared to 1 15 parts *per million* for the standard cigarette (Fig 3)

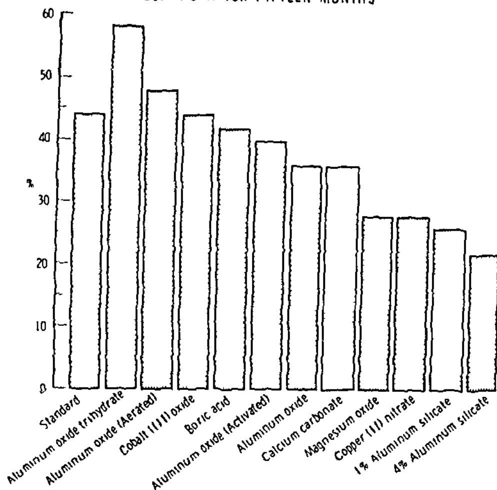


Fig 2

In each experiment 50,000 cigarettes were smoked All the cigarettes were smoked on a standard smoking machine previously described *which puffed three times a minute (28) The results of these experiments* were compared to standard cigarette condensate from the same smoking machine In two instances, calcium carbonate and copper (II) nitrate, the cigarettes were smoked in addition on a smoking machine puffing each cigarette individually once a minute These condensates were compared to standard cigarette condensate from the same smoking machine

The biological results from condensates obtained from the three times a minute smoking machine indicate an increase in activity for cigarettes to which aluminum oxide trihydrate and moisturized aluminum oxide have been added (Fig 2) The materials were applied in 50 per cent concentration with No 5 Camel hair brushes three times a week to the whole back of Swiss (Millerton) female mice The mice were shaved whenever necessary There was a suggestive decrease in biological activity in cigarettes treated with copper nitrate, magne-



Fig 5

ever in a previous study where tobacco smoke condensate was separated into basic, acidic and neutral fractions and then recombined no loss of activity was observed (28)

With a new technique, using distribution between organic solvents followed again by column chromatography one could remove polycyclic hydrocarbons more carefully to nearly 0 0 ppm (12) This separation is now under way for later biological testing All subfractions of the chromatographed nitromethane extract are tested by chromatography on acetylated paper The polynuclear hydrocarbon containing fractions are recombined and rechromatographed This results in a fraction smaller than one per cent which contains all polycyclic hydrocarbons The other fractions are combined and will be used for final testing

Side Stream Smoke Chemical analysis of the side stream smoke showed a marked increase in benzo(a)pyrene (4 7 ppm), a finding in agreement with that of *Kotin & Falk* (15) (*Fig 5*) This indicates again that the formation of higher polycyclic hydrocarbons is a result of incomplete combustion and that more complete combustion as does take place during the puffing of cigarettes forms less polycyclic hydrocarbons The differences in combustion temperatures during the resting and puffing phase are not very marked A cigarette burns at rest at a temperature of 835° C and during puffing has an average temperature of 884° C (22)

Puff Frequency Benzo(a)pyrene determinations on cigarettes puffed once twice and three times a minute showed a tendency to increase in benzo(a)pyrene content from 1 15, 1.25 and 1 35 respectively The

(11, 12) The biological results from the smoking machine puffing once a minute show differences for both additives compared to the standard tar (Fig 4) At the end of 15 months the standard condensate shows a tumor yield of 44 per cent papillomas and 14 per cent cancers The cigarette treated with calcium carbonate yielded a condensate that gave 18 per cent papillomas and 2 per cent cancers The one treated with copper (II) nitrate was still lower yielding 6 per cent papillomas and 2 per cent cancers The much lower activity of the condensate from cigarettes treated with copper (II) nitrate also was indicated by the fact that the first papillomas appeared five months later than that of the standard condensate There can be no doubt, therefore, that at least with some additives a significant reduction in biological activity can be obtained Work is now in progress to determine precisely what other substances may be lowered besides polycyclic hydrocarbons since whatever reduction is observed for benzo(a)pyrene and other so far identified carcinogenic hydrocarbons is not sufficient to account for the decrease in biological activity The reason why the decrease in activity is more marked in the once a minute smoking machine compared to the three times a minute machine may be that in the latter instance the amount of aerosols that settles in a cigarette is so great as to overwhelm the catalytic activity of an additive As far as the standard cigarettes are concerned, the biological activity of condensate from the three times a minute smoking machine is not significantly different from that of the one time a minute smoking machine The present biological results with additives are to be regarded as preliminary findings Large scale additional biological investigations are necessary to substantiate the present data

Reduction of Polynuclear Aromatic Hydrocarbons in Cigarette Smoke Condensate The concept that polycyclic hydrocarbons play an important role in tobacco carcinogenesis is underlined in an experiment where the polycyclic hydrocarbons were removed from tobacco smoke condensate to the extent of lowering the benzo(a)pyrene to 0 1 ppm which compares to the standard value of 1 15 ppm We utilized an old technique of partitioning in the neutral portion (28) All fractions besides the so called B fraction (hexane eluate) yielded the material for biological testing Biological studies with this material now in progress, after 11 months shows only one out of 50 Swiss mice developed a papilloma compared to the standard condensate which developed 14 papillomas and 3 cancers In this chemical separation, of course, we have not only reduced the amount of polycyclic hydrocarbons, but also some other compounds of the neutral fraction, since only 95 per cent of the neutral portion could be used in the recombination of the other portions It is, of course, conceivable that during the chemical separation, the materia lost biological activity (use of alkali and acid) How-

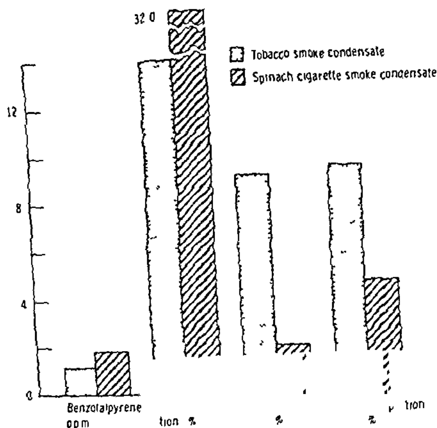


Fig 6

Short term tests during which various fractions of tobacco smoke condensates are applied to mouse skin have shown that the sebaceous gland activity as well as the degree of hyperplasia are not due to the polycyclic hydrocarbons in the amount present in tobacco smoke condensate but rather are due to several other groups of substances in tobacco smoke condensate (21) Such tests in 50 per cent concentration have shown positive sebaceous gland and hyperplasia activity for the nicotine free basic portion as well as for the acidic and neutral fractions of tobacco smoke condensate (21 28) With more diluted solutions (33 per cent) we have found the highest short term activity of the major condensate fractions with the phenolic and the acidic fractions Table 1 We also found that if the phenolic fraction is removed from tobacco smoke condensate the remaining condensate has virtually lost all of its short term activity when tested in 33 per cent concentration The biological activity of total tobacco smoke conden

difference between each consecutive value is within the experimental deviation which is ± 0.1

Butt Length The main stream of the second half of a cigarette contains less polycyclic hydrocarbons (benzo(a)pyrene 0.85 ppm) than the first half (benzo(a)pyrene 1.85 ppm) per gram condensate, obtained under standard smoking conditions using the full automatic Ethel Mark VII smoking machine (25). This result opposes formerly published data (15, 16). Therefore, analyses have been repeated several times, using the tracer-technique for benzo(a)pyrene (11) and still confirmed our first finding. Since such data might be indicative for the way in which polynuclear hydrocarbons are formed during the combustion of a cigarette, we are presently engaged in the determination of the amount of polynuclear hydrocarbons in condensates from different sections of the cigarette. In this respect we found in our standard cigarette (85 mm) which we shortened to 54 mm and smoked down to a butt of 23 mm as usual, the amount of 1.85 ppm which is within experimental deviation, the same amount obtained from the condensate of the first 31 mm of an 85 mm standard cigarette. These results perhaps indicate that the condensed smoke particles within the cigarette influence the combustion process and thus the forming of combustion products. Further investigations are in process.

Other Plant Products Analysis of the condensates of non-tobacco cigarettes made of different vegetable fibers and spinach showed a higher content in these materials of polynuclear hydrocarbons (benzo(a)pyrene, 2.3 and 1.9 ppm respectively) as compared to tobacco cigarette condensate which may be a result of less complete combustion of the products and due to differences in the nature and composition of the precursors of these materials. The data for the tobacco and spinach cigarettes are compared in Fig. 6.

In summary, the formation of polynuclear aromatic hydrocarbons regarded as a major group of initiating carcinogens in tobacco smoke condensate, seems to be a result of incomplete combustion of tobacco. Steps that could lead to more complete combustion of tobacco should lead towards a reduction in these substances. Some additives may work towards this end.

PROMOTING COMPOUNDS

Adding the number of carcinogenic polycyclic hydrocarbons identified in tobacco smoke condensate and comparing it to the established biological activity of this material indicates that they can account for only a small part of the total activity (24). There have to be in smoke condensate either other initiating carcinogens or promoting substances, or both. Evidence now on hand indicates that tobacco smoke condensate does not only contain initiating carcinogens, but also promoting substances.

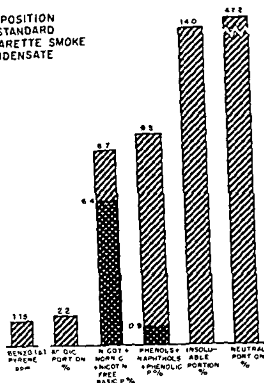


Fig 7

tar in determinations of parts per million and percentages, and we suggest that in future comparisons of chemical constituents of different types of smoke condensate, all of these fractions be determined and listed and, in addition, maybe the value of phenol itself

At the present time we are analyzing the portion which has practically all the polycyclic hydrocarbons and accounts for less than 2 per cent of the total condensate, for the presence of promoting substances which must be of a different type than those described (Fig 1) Possibly some of these are long chained unsaturated hydrocarbons such as polyterpenes At any rate, we know from our biological experiments that other carcinogenic or promoting materials must be present also in this fraction This fraction also had positive short term as well as long term activity which cannot be accounted for by the presence of so far herein known polycyclic aromatic hydrocarbons Short term tests have shown that the biological activity of subfractions of fraction B (Figure 1) of the neutral tar has been placed by solvent distribution between cyclohexane and nitromethane into the nitromethane fraction We now have a neutral constituent of only 0 15 per cent of the total smoke condensate where the benzo(a)pyrene content has been enriched to 750 ppm and

sate minus the phenolic fraction on the basis of long term studies remain to be carried out Short term tests with spinach cigarettes added to our knowledge of promoting substances In the spinach cigarette both the basic and phenolic fractions are low These results are consistent with the belief that in the short term test with cigarette smoke condensate substances other than polycyclic hydrocarbons are responsible for positive results

Boutwell has demonstrated some promoting activity for phenol and phenolic fractions in 10 per cent and 5 per cent concentration (2) The phenol results have been confirmed in our laboratory using a single dose of 75 μg of 7 12 dimethylbenz(a)anthracene as well as repeated applications of benzo(a)pyrene in a 0 005 per cent concentration as initiator For both substances 10 per cent phenol proved to be a potent promoting substance The phenol fraction constitutes about 9 to 10 per cent of tobacco smoke condensate, about 10 per cent of which are phenols (27) Promoting activity was also shown for the acidic and phenolic fraction (10 per cent acetone solution) but none for the neutral fraction after six months using 3 times weekly painting applications of 0 005 per cent benzo(a)pyrene as initiator

TABLE 1
Short Term Tests

Condensate Fractions (33 %)	Relative Activity
Cigarette	+++
Acidic	++
Basic	+
Neutral	
Phenolic	+++
Cig minus Phenol	+

Long term biological tests already completed have shown that the nicotine free basic portion of tobacco smoke condensate has some carcinogenic activity (28) It has also been shown that the acidic plus the neutral fraction together have a greater biological activity than either of these fractions alone (28) Gellhorn has shown promoting activity for total tobacco smoke condensate (6) Significant promoting activity appears in the phenolic fraction and particularly with phenol itself Roe has shown promoting activity for the phenolic fraction using 300 gammas DMBA as initiator (19) We would predict that if the phenolic and acidic fractions of tobacco smoke condensate are significantly reduced, the total biological activity of tobacco smoke condensate would decline Long chain acids, as present in the acidic fractions, may also have promoting activity (13, 27) It is pertinent in presenting the chemical constituents of tobacco smoke condensate to list not only benzo(a)pyrene, but also important subfractions since we regard the total biological activity a result of all these fractions In Fig 7 we list the chemical constituents in tobacco smoke condensate for a standard cigarette

(3) A reduction of promoting substances, such as the phenols and long chain acids, through selective filtration is more likely than that of polycyclic hydrocarbons because they are present in larger concentrations, and what is more important, they have acidic functional groups. Since polyphenols are regarded as a major precursor of the phenols, and since air cured tobacco contains definitely less polyphenols than flue cured tobacco an additional way of reducing phenols in tobacco smoke condensate may be at hand (14).

SUMMARY AND CONCLUSIONS

(1) The basic proof that smoking is a cause of certain types of human cancer rests on epidemiological studies. Such studies have established that smoking, particularly of cigarettes, represents a cause of cancer of the lung and that smoking also represents a cause of cancer of the mouth, larynx and esophagus.

(2) Animal evidence underlines the epidemiological data in that it demonstrates that tobacco smoke condensate considered to be carcinogenic to man is proved to be carcinogenic to laboratory animals.

(3) Biological and chemical evidence has demonstrated that the carcinogenic activity is due to the presence of initiating as well as promoting substances. A reduction in either of these groups would lead to a reduction in carcinogenic activity.

(4) Steps have been listed which should reduce the risk of lung cancer, for smokers who cannot give up or drastically reduce their smoking habits. These included measures leading to a reduction in smoke condensate exposure for the individual smoker and in steps directed towards reducing initiating carcinogens and promoting substances in tobacco smoke condensate.

(5) Success along these lines should be followed by a reduction in the incidence of those cancers related to smoking.

REFERENCES

1. *American Cancer Society* Annual Report 1958 p 20
2. *Boutwell R K & Bosch D K* The tumor promoting action of phenol and related compounds for mouse skin Cancer Res *19* 13 24 1959
3. *Burney L F* Lung cancer and excessive cigarette smoking Dr Little's reply CA Bull Cancer Progr 8 44 1958
4. *Doll R & Hill A B* Lung cancer and other causes of death in relation to smoking second report on mortality of British doctors Brit M J 2 1071 1081 1956
5. *Dorn H F* Tob[illegible] from cancer and other diseases
6. [illegible] garette Tobacco Tar Cancer Res
7. [illegible] co smoking and cancer of lung [illegible]
9. [illegible]

where most of the short term activity resides We are now in the process of separating this fraction *into the polycyclic hydrocarbon fraction* and other materials

PREVENTIVE MEASURES

It has been claimed that one could not establish the carcinogenic activity of a given substance unless one had isolated specific carcinogens This is an ill founded view If a given material produces cancer for a given animal tissue this proves by definition that this material is carcinogenic and whether or not one can identify the specific carcinogen does not alter this fact Our reason for searching for the specific carcinogens or co carcinogens in tobacco smoke condensate has not been to give more credence to the human and animal findings but rather to permit upon identification of these substances to study their possible reduction Within our present knowledge we can account for a majority of the established biological activity of tobacco smoke condensate *through a combination of initiating carcinogens and promoting* substances

The biological results of different smoke condensates suggest the presence of *initiating carcinogens* as well as *promoting substances* The next step must be to attempt a reduction of both these groups Proper advance in either direction should reduce the total activity of tobacco smoke condensate

As stated the major purpose of animal evidence is to define the mechanism for a better understanding of carcinogenesis and in particular to define the specific substances responsible for the carcinogenic activity In view of the data at hand the following preventive measures short of giving up smoking altogether which obviously would be the most complete measure are in order

(1) Reduction in exposure to smoke condensate through (a) Modification of smoking habits (b) Cigarettes with resulting lower content of smoke condensate (c) Avoidance of deep inhalation smoking cigarettes to the end and of drawing on a cigarette too frequently

(2) Further attempts to reduce the initiating carcinogens through the use of additives Present attempts in this direction are promising Such additives may not only decrease polycyclic hydrocarbons but possibly also promoting substances as found for the phenolic fraction from cigarettes *with calcium carbonate and copper(II)nitrate* as additives Attempts have also been made to study precursors of the polycyclic hydrocarbons with the hope that some precursors are particularly susceptible to form these substances (29) We find however that most organic materials yield polycyclic hydrocarbons during pyrolysis especially above 700° C Also it does not seem possible to remove selectively the polycyclic hydrocarbons through filtration

AUS DER I UND II UNIVERSITÄTS-FRAUENKLINIK HELSINKI
(VORSTÄNDE PROF DR AARNO TURUNEN UND PROF DR PAAVO VARA)

BEOBACHTUNGEN ÜBER DAS VORKOMMEN VON SCHAUMZELLEN IM ENDOMETRIUMSTROMA BEI HYPERPLASIE

Von

CLAËS V. NUMERS und USKO NIEMINEN

Eingegangen 9 xi 60

Abgesehen von den für die Malignität characteristischen Veränderungen im Endometrium sind im Zusammenhang mit Carcinoma corporis uteri gelegentlich im Stroma Zellen beobachtet worden, die weitgehend von ihrer Umgebung abweichen. Manche Autoren haben diese Zellen als Xanthomzellen angesprochen, während sie nach anderen wieder um hinsichtlich Bau und sonstigen Eigenschaften von diesen abweichen, weshalb sie u. a. als foam cells, Schaumzellen, Pseudoxanthomzellen usw. bezeichnet worden sind. Die genannten Zellen wurden im Stroma des Endometriums verhältnismässig selten angetroffen und dann fast ausnahmlos im Verein mit Korpuskarzinom.

Harris (1958) fand derartige Zellen in 16 von 150 untersuchten Gewebsproben von Korpuskarzinom. Im Zusammenhang mit anderen pathologischen Veränderungen des Endometriums konnte er keine beobachten, dagegen in zwei von 400 untersuchten Zervixpolypen. Harris bezeichnet die Zellen als "foam cells".

Eine ähnliche Untersuchung haben auch *Krone & Littig* (1959) ausgeführt, wobei sie zu dem Resultat kamen, dass Pseudoxanthomzellen [illegible]

[illegible] dass diese Zellen kein Glykogen enthielten, und dass sie bei Polarisation keine Doppelbrechung hervorrufen. Sie können daher nicht als Xanthom-, sondern nur als Pseudoxanthomzellen gelten.

Nach der Auffassung von *Pick* (1908) werden Xanthomzellen auch im Pyometrium angetroffen. *Schiller* (1927) wiederum ist anderer Meinung, denn bei seinen umfangreichen Untersuchungen konnte er weder in alten noch in frischen Pyometrien derartige Zellen finden. Auch in der Literatur wird nichts berichtet, was die Beobachtung von Pick bestätigen könnte.

Wie aus dem obigen hervorgeht, werden die Schaumzellen fast ausnahmlos im Endometrium des Korpusteils und auch hier nur sporadisch

8 *Hammond E C & Horn D* Smoking and death rates—report on forty four months of follow up of 187 783 men 1 Total mortality J A M A *166* 1159 1172 1958
Smoking and death rates—report on forty four months of follow up of 187 783 men 2 Death rates by cause Ibid *166* 1294 1308 1958
9 *Heller J R* If you re worried about cancer—advice from authority U S News & World Report *46*(21) 60 & 62 May 25 1959
10 *Hilleboe H E* Smoking and Health The New York Times (August 22) 1958
11 *Hoffmann D & Wynder E L* On the isolation and identification of polynuclear aromatic hydrocarbons Cancer *13* 1062 1073 1960
12 *Hoffmann D & Wynder E L* Short Term Determination of Carcinogenic Aromatic Hydrocarbons Anal Chem *32* 295 1960
13 *Holsti Paul* Tumor Promoting Effects of some Long Chain Fatty Acids in Experimental Skin Carcinogenesis in the mouse Acta path et microbiol scandinav *46* 51-58 1959
14 *Johnstone R A W & Plimmer J R* The Chemical Constituents of Tobacco and Tobacco Smoke Chem Rev *59* 885 936 1959
15 *Kotin P & Fall H L* The Role and Action of Environmental Agents in the Pathogenesis of Lung Cancer 2 Cigarette Smoke Cancer *13* 250-262 1960
16 *Lindsay A J* Tobacco Smoke Brit M J *1* 506 1959
17 *Orris L Van Duren B L Kosak A I Nelson N & Smith F L* The Carcinogenicity for Mouse Skin and the Aromatic Hydrocarbons Content of Cigarette Smoke Condensates J Nat Cancer Inst *21* 557 561 1958
18 Press Notice (No 1233) Prepared by Netherlands Ministry of Social Affairs and Public Health The Hague Netherlands 1952
19 *Roe F G C* The Promoting Activity of Phenolic Fraction of Cigarette Smoke Condensate Brit J Cancer *13* 623 633 1959
20 *The State Medical Research Council of Sweden 1958*
21 *Suntzeff V Croninger A B Wynder E L Cowdry E V & Graham E A* Use of sebaceous gland test of primary cigarette tar fractions and of certain non carcinogenic polycyclic hydrocarbons Cancer *10* 250 254 1957
22 *Touey G P & Mumpower R* [illegible]
perature of Cigarettes
23 World Health Organization [illegible]
of the lung p 4 5 1960
24 *Wynder E L & Hoffmann D* A study of tobacco carcinogenesis VII The role of higher polycyclic hydrocarbons Cancer *12* 1079 1086 1959
25 *Wynder E L & Hoffmann D* Some practical aspects of the smoking lung cancer problem New England J Med *262* 540 545 1960
26 *Wynder E L Kopf P & Ziegler H* Study of tobacco carcinogenesis II Dose response studies Cancer *10* 1193 1200 1957
27 *Wynder E L & Hoffmann D* Cancer in press
28 *Wynder E L & Wright G* A study of Tobacco Carcinogenesis I The primary Fractions Cancer *10* 255 271 1957
29 *Wynder E L Wright G F & Lam J* A Study of Tobacco Carcinogenesis VI The Role of Precursors Cancer *12* 1073 1078 1959

Die in der Schleimhaut des Corpus Uteri anzutreffenden Makrophagen teilt *Teyrler* (1937) in vier Gruppen ein, die er nach den Stoffen benennt, die sie phagozytiert haben: 1. Die *Cytophagen* sind Zellen, die Erythrozyten gefressen und zersetzt haben (Erythrophagen, *v. Numers* 1942). 2. die *Siderophagen*, in denen Haemosiderin angetroffen wird (v. Numers 1942). 3. die *Lipophagen*, die neutrales Fett phagozytieren, und 4. die *Mucophagen*, die saure, offenbar zu den Polysacchariden zählende Stoffgruppen fressen und aufspeichern. Teyrler bemerkt, dass alle die oben genannten Stoffe auch in ein und derselben Zelle gefunden werden können.

EIGENES MATERIAL

Im wissenschaftlichen Labor der Universitäts-Frauenklinik in Helsinki sind unter den untersuchten pathologisch-anatomischen Proben seit dem Jahre 1938 in 12 hyperplastischen Endometrien Schaumzellen gefunden worden, und ferner wurden derartige Zellen noch bei einigen Korpuskarzinomen sowie in einem Zervixpolyp angetroffen. Von den oben genannten 12 Patientinnen standen 10 im Alter von 41–64 Jahren, nur zwei waren jünger, die eine 39 und die andere 27 Jahre alt.

Die Proben waren meistenteils bei Ausschabungen gewonnen worden, nur einige wenige bei Operationen. Alle waren nach Formalinfixation und Alkohol-Xylolbehandlung in Paraffin eingebettet worden. Mit dem Mikrotom wurden von Paraffinblock für die Färbung 4 μ starke Schnitte gemacht, die alle nach van Gieson und mit Hämatoxylin-Eosin gefärbt wurden. Ein Teil der Proben w[illegible] der *Perjod*säure-Schiff-Reaktion [illegible] *Fool*) und nach dem Verfahren

Die Menge der Schaumzellen ist in diesen Präparaten recht unterschiedlich. In manchen sieht man nur vereinzelte Zellen oder kleine Gruppen (Fig. 1), während die Zellen in anderen Proben wiederum grössere Haufen bilden (Fig. 2). Die Schaumzellen, die in allen Schichten des Stromas vorkommen, sind grösser als die anderen im Stroma [illegible] sehr verschieden, gewöhnlich sind [illegible] Umgebung unterscheiden sie sich [illegible] ...wiesene Farbbarkeit (Fig. 3). Das Protoplasma ist hell und schaumig, es scheint gewissermassen aus einem feinen Netz oder winzigen Bläschen zu bestehen, und teilweise führt es reichlich kleine Granula. Stellenweise sieht man in oder zwischen den Zellen runde Öffnungen oder Vakuolen. Aus der Struktur zu schliessen haben die Zellen Fett enthalten, das jedoch bei der Behandlung der Proben verschwunden ist. Da und dort sieht man, dass die Zellen Blutpigment enthalten, das am deutlichsten in den mit Hämatoxylin-Eosin gefärbten Präparaten hervortritt (Fig. 4). Der Zellkern ist zumeist klein und stark gefärbt, oft exzentrisch gelegen, seine Form kann sehr verschieden sein, meistens ist er rund oder oval. Die Kernmembran ist ziemlich dick. Zuweilen kommen zweikernige Schaumzellen vor. Bei der Perjodsäure

angetroffen, während sie in der Schleimhaut der Zervix als grösste Seltenheit gelten können (*Chiari* 1955, *Harris* 1958, *Krone & Lüthy* 1959 und *Schiller* 1927).

Dubs (1923) beschreibt zwei Fälle, wo Xanthomzellen im Korpuskarzinomgewebe vorgefunden wurden. In beiden Fällen konnte bei der betreffenden Patientin keine Hypercholesterinämie festgestellt werden, weshalb die Zellen seines Erachtens nicht dadurch bedingt sein konnten, wie früher angenommen worden war (*Pinkus & Pick* 1908). *Lubarsch* (1918) meint, dass die Ursache der begrenzten Xanthombildungen keine generelle sei, sondern Folge einer lokalen Störung des Cholesterinstoffwechsels.

Nach seinen weitläufigen Ausführungen über das Vorkommen der Xanthomzellen im Endometrium und ihre Herkunft kommt *Schiller* (1927) zu folgenden Schluss: „Zusammenfassend lässt sich sagen, dass wir es in unseren Fällen mit einer örtlichen Cholesterinspeicherung in Zellen des cytogenen Gewebes zu tun haben, die auf Grundlage einer allgemeinen Bereitschaft: physiologische Hypercholesterinämie infolge zahlreicher Geburten und infolge des Klimakteriums, verbunden mit örtlichen Ursachen, wahrscheinlich Abhebung von Schleimhautfalten, auftritt."

Spivack (1932) hat im hyperplastischen Gewebe der Uterusschleimhaut grosse mononuklare Zellen mit viel Protoplasma und exzentrisch gelegenen Kern gefunden. Sie beschreibt die fraglichen Zellen jedoch nicht genauer und sagt auch nichts über ihre Herkunft. Spivack gebraucht für diese Zellen auch keine besondere Bezeichnung.

Gillman (1941) hat die Beobachtung gemacht, dass bei Metropathien im Stroma Fett angereichert wird. *Black, Heyns & Gillman* (1941) stellten fest, dass bei zwei kastrierten Frauen, denen Oestrogen verabreicht wurde, das Fett in den Stromazellen zunahm. Auf Grund dessen kommen sie zu der Ansicht, dass möglicherweise das Oestrogen beim Auftreten von Schaumzellen im Stroma eine Rolle spielen könnte.

Der heutigen Auffassung gemäss sind die Ursachen für das Entstehen von Schaumzellen in erster Linie örtlicher Natur. Bei Entzündungen und auch in Zusammenhang mit Drüsentumoren treten Hyperämie und Gewebsveränderungen in dem erkrankten Gebiet ein. Infolge von fettiger Degeneration der Leukozyten und Gewebszerfall entstehen Fettstoffe, die von den phagozytierenden Zellen gefressen werden. Demzufolge wachsen diese an, ihr innerer Bau erfährt Veränderungen, das Protoplasma wird schaumig, der Kern schrumpft und wandert zur Peripherie, und die Zellen nehmen das Aussehen von Xanthomzellen an.

Im Stroma der normalen Uterusschleimhaut werden in allen Phasen des Menstruationszyklus freie, makrophagenähnliche, phagozytierende Zellen angetroffen, die nicht selten Blutpigment enthalten. Die Makrophagen vermehren sich nach dem Gewebszerfall bei der Menstruation. Diese Makrophagen gehen mindestens teilweise aus den Reticulumzellen des Stromas hervor (*v. Numers* 1942).



Figs 4-6

Fig 4 Schaumzellen mit Blutpigment im Plasma Haem Eosin × 500
Fig 5 Perjodsäure Schiff positive Granula im Plasma der Schaumzellen Perjod

[illegible] der Faser in
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Figs 1 3

Fig 1 Zerstreut im Stromagewebe erscheinende einzelne Schaumzellen Haem Eosin × 125

Fig 2 Grössere Gruppen von Schaumzellen im Stromagewebe des hyperplastischen Endometriums *Haem Eosin* × 125

Fig 3 Zahlreiche Schaumzellen mit hellem teilweise granuliertem Plasma Haem Eosin × 500

ZUSAMMENFASSUNG

Die Verfasser haben abweichend von den früher in der Literatur mitgeteilten Untersuchungen festgestellt, dass Schaumzellen auch in hyperplastischem Endometrium vorkommen. Die Schaumzellen entsprechen hinsichtlich Struktur und sonstiger Eigenschaften den in der normalen Schleimhaut anzutreffenden Makrophagen, weshalb sie als die gleichen Zellen angesehen werden. Zweifellos steht eine extreme Form dieser Zellen in Frage.

SUMMARY

The authors have established the occurrence of foam cells in the stroma of the hyperplastic human endometrium, earlier reported only in connection with adenocarcinoma of the endometrium. Concerning their structure and other qualities the foam cells correspond to the macrophages of the normal endometrium and obviously represent an extreme form of these cells.

LITERATUR

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Daus J. Xanthomzellen in der Uterusschleimhaut bei Funduscarcinom. Zentralbl. allg. Path. 34: 145, 1923.

Feyrter F. Über den zelligen Bestand des Stroma der menschlichen Corpusmucosa. Arch. f. Gynäkol., 190: 47, 1957.

[illegible] Body of the Uterus.

[illegible] Vorkommen von Schaumzellen im Stroma von Adenocarcinomen des Corpus Uteri. Arch. f. Gynäkol. 191: 432, 1959.

Lubarsch O. Generalisierte Xanthomatose bei Diabetes. Deutsche med. Wchschr. 44: 484, 1918.

Numers C. Über die Zellformen des Stromagewebes der menschlichen Gebärmutterschleimhaut. Acta Obst. et Gynec. Scand. 22: Suppl. 3, 1942.

Pick L. Über besondere Strukturen in alten Eitertuben. Berlin. klin. Wchschr. 37: 1697, 1908.

Pinkus F. & Pick L. Zur Struktur und Genese der symptomatischen Xanthome. Deutsche med. Wchschr., 34: 1426, 1908.

Schiller W. Über Xanthomzellen im Uterus. Arch. f. Gynäkol. 130: 346, 1927.

Spitack M. The Histopathology of the Uterus in Relation to the So Called Essential or Idiopathic Uterine Bleeding. Surg. Gynec. and Obst., 55: 733, 1932.

Schiff-Färbung sieht man im Plasma der Schaumzellen kleine, sich positiv anfärbende Granula (Fig. 5). Im Bereich der Schaumzellen kommen bei der Silberimprägnierung deutlich weniger Präkollagenfasern zum Vorschein als sonst im Stromagewebe (Fig. 6). Beim Wachsen schieben die Zellen die Fasern vor sich her. Da am Rand der Zellgewebe jedoch keine Fibrillenhaufen zu sehen sind, muss ein Teil der Präkollagenfasern atrophiert und verschwunden sein.

BESPRECHUNG

Den früheren Untersuchungen gemäss werden Schaumzellen im Stroma des Endometriums nur im Verein mit Korpuskarzinom angetroffen, und nur ganz ausnahmsweise im Zellgewebe von Zervixpolypen oder Zervixkarzinom. Soweit uns bekannt ist, sind bis jetzt keine Mitteilungen über Vorkommen von Schaumzellen im hyperplastischen Endometrium publiziert worden. Spivack erwähnt in ihrer Veröffentlichung nebenbei grosse mononukleare Zellen, die sie in hyperplastischem Korpusendometrium gefunden hatte, ohne jedoch Genaueres über ihren Befund zu berichten. Gillman hat Vermehrung des Fetts im Stroma bei Metropathien beobachtet, und Black, Heyns sowie Gillman verweisen auf die eventuelle Rolle des Oestrogens in der Ätiologie der Schaumzellen.

Wie aus der Beschreibung unseres eigenen Materials hervorgeht, werden Schaumzellen gelegentlich auch in hyperplastischem Korpusendometrium gefunden, allerdings nur relativ selten, so dass also von keiner mit der Hyperplasie regelmässig verknüpften Erscheinung die Rede sein kann. Trotz ihrer Seltenheit sprechen diese Befunde aber zugunsten der oben erwähnten Vermutung von Black, Heyns und Gillman. Bis auf zwei Ausnahmen standen die betreffenden Patientinnen alle dem Klimakterium nahe oder hatten es bereits überschritten.

Die heutzutage vorherrschende Auffassung von der Ätiologie der Schaumzellen steht im Einklang mit den von v. Numers und Teyrter gemachten Beobachtungen. Das in den Schaumzellen oft gefundene Blutpigment stellt unzweifelhaft phagozytierten Stoff dar, was augenscheinlich auch betreffs der nachgewiesenen schleimartigen Substanzen zutrifft. Die Struktur des Zytoplasmas spricht wiederum dafür, dass die Zellen Fettstoffe gefressen haben. Die Schaumzellen sind hinsichtlich ihrer Eigenschaften und ihres Baus der von v. Numers und Teyrter beschriebenen Makrophagen so ähnlich, dass sie als eine extreme Form dieser Zellart angesehen werden müssen. Der die Hyperplasie kennzeichnende, von Ausbleiben der Desquamation bedingte lange Bestand des Korpusendometriums sowie die allmählich einsetzenden regressiven Veränderungen bieten offenbar gelegentlich günstige Verhältnisse für das Auftreten von Makrophagen des Schaumzellentypus.



Fig 1
Low power magnification of the tumour showing three cell zones in the tumour-islands In the central part cystic cavities are formed Cold absolute acetone fixation (H & E × 95)

dehydration the specimens were embedded in a mixture of n butyl and methyl meta-crylate (9 1) to which 1 per cent benzoyl peroxide had been added as a catalyst Sections were cut on a *Sjostrand* microtome using glassknives and were examined with a Philips EM 100 B

RESULTS

Lightmicroscopic Studies

In most epithelial tumour islands three cell-zones can be distinguished a peripheral zone composed of a single layer of cells, an intermediate zone composed of a varying number of cells, and a central zone in which one or often several cystic cavities can be found (Fig 1)

The peripheral or marginal cell layer usually consists of a single row of columnar cells resembling the inner enamel epithelium of the enamel organ Intercellular cytoplasmic bridges are seen crossing the irregular *spaces between adjacent cells*

The *intermediate* zone is composed of anastomosing stellate cells and has a superficial resemblance to the stellate reticulum of the enamel organ This zone often forms the main part of the single tumour-island Desmosomes can easily be demonstrated where the cells are in close contact with each other (Fig 2)

The cells occupying the *central zone* of the island are polyhedrical, but vary in shape and size often being rather large (Fig 2) Areas with intimate relations between cells alternate with areas of loose texture

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THE ULTRASTRUCTURE OF THE SIMPLE AMELOBLASTOMA

By

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Received 15 xii 60

The ameloblastoma (or adamantinoma) is a benign odontogenic epithelial tumour having no properties of producing hard dental tissues It occurs within the jawbones, the most frequent site being the lower molar region The ameloblastomas show considerable variation in histomorphology and have been classified into several types one of which is the simple ameloblastoma In this type the epithelial tumour tissue is arranged in irregular islands or acanthomatous masses or it may show a plexiform pattern The tumour islands are embedded in a mature connective tissue stroma The histomorphology of the simple ameloblastoma has been described in great detail in several light microscopic studies (*Bernier* 1959, *Lange*r 1958, *Small & Waldron* 1955 and *Thoma* 1951) whereas no electron-microscopic study of this tumour seem to have been published The present investigation is concerned with the ultrastructure of a simple ameloblastoma as revealed by electronmicroscopic examination

MATERIAL AND METHODS

The tumour tissue examined was removed during operation of an ameloblastoma occurring in the lower jaw of a 40 year old woman The tumour presented itself as a swelling the size of a walnut located in the left premolar and molar region The swelling had increased slowly during the last six month prior to admission X ray examination revealed a multilocular cystic lesion with a scalloped outline On histology the tumour proved to be a simple ameloblastoma and the tumour containing area of the mandible was resected

For examination in light microscope the tissue was fixed in saline formaldehyde (4 per cent) in cold (4°) absolute acetone and in modified *Davidson*s fixation fluid After embedding in paraffin 4 6 micron thick sections were cut and stained with haematoxylin eosin *van Gieson*s connective tissue stain *Lendrum*s phloxin tartrazin, unstained sections were used for phasecontrast microscope studies

Tissue to be examined in electronmicroscope was obtained during the operation and was fixed immediately either in 1 per cent buffered OsO_4 to which sucrose has been added (*Caulfield* 1957) or in potassiumbichromate (*Dalton Felix* 1954) After

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Fig 3

Electron micrograph showing the peripheral cells separated by irregular intercellular cleft N nucleus n nucleolus M mitochondria D attachment plaques BM basement membrane F fibroblast OsO_4 fixation × 10 300



Fig. 2
Phase contrast photo-micrograph showing the three cell zones of the simple ameloblastoma. At the bottom cylindrical (peripheral) cells, in the mid zone star-shaped cells showing desmosomes (arrows) and at the top of the photomicrograph the large polyhedrical cells with intracellular cavities (S). OsO_4 fixation. × 900

Apart from this typical picture some epithelial islands composed of only peripheral and intermediate zones and few islands composed almost exclusively of stellate cells could be seen.

Whereas the nuclei in the peripheral and intermediate cells form a comparatively great part of the cell-body, the nuclei of the central cells are quite small and condensed. The nuclei contain one or several nucleoli, those of the peripheral nuclei having the most distinct ones. In the cytoplasm of many of the columnar cells a PAS-positive pyroninophile and metachromatic substance was demonstrated. This substance was particularly obvious in the basal part of the cytoplasm. The cytoplasm of the central cells lining the cystic spaces shows a marked eosinophilia.

The epithelial tumour-islands are embedded in a collagenous con-



Fig 5
Intermediate cells from a tumour island Some cells are closely approximated by means of interdigitating cytoplasmic projections At points of contact attachment plaques are present In the cytoplasm fibrillar elements (F), mitochondria (M) and circular profiles (P) OsO_4 × 4 500

(Fig 3) Below or peripheral to this membrane a layer of coarse, spongiform material with the same electron density as basement membrane can be seen The connective tissue fibers and the fibroblasts may be located close to and parallel with the basement membrane in those areas which lack the layer of spongiform substance

The columnar or *peripheral cells* contain many round or ovoid mitochondria many of which are located in the vicinity of the poles of the nuclei The mitochondria measure 0 25 micron in diameter (Fig 3) and show a triple layered outer membrane and a few inner membranes or cristae likewise triple-layered In the cytoplasm of some peripheral cells a complex system of irregular vacuoles or canaliculi is noted (Fig 4) These vacuoles are to some extent in communication with each other They seem to be bordered by a thin membrane having minute granules attached to the outer surface The vacuoles contain an almost homogeneous, somewhat flocculent material of moderate electron density



Fig 4
Part of the infranuclear cytoplasm in a peripheral cell Conspicuous in the cytoplasm is a system of cavities or canaliculi (S) containing a substance of moderate electron density I intercellular cleft OsO_4 fixation × 26 900

nective tissue stroma with a moderate number of cells (predominantly fibroblasts) and bloodvessels (Fig 1) The peripheral columnar cells rest upon a well defined basement membrane adjacent to which an eosinophilic hyaline zone may be seen The histomorphological picture described here is thus typical of a simple ameloblastoma

Electronmicroscopic Studies

A continuous basement membrane, 350 600 Å thick, separates the connective tissue stroma from the peripheral cells of the tumour islands



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Fig 6

a *Micrograph illustrating the appearance of many attachment zones (D) cut in* a plane perpendicular to the surface of the cell Tonofilaments (F) coming from the cytoplasm attach themselves of the desmosomes or attachment plaques (D) A layer of low electron density separates the attachment plaques OsO_4 fixation × 13 250

b In the center of the micrograph a group of small circular profiles varying in size These presumably represent *Golgi* vesicles OsO_4 fixation × 20 600

c Mitochondria in the cytoplasm of cells from the intermediate zone The mitochondria show three layered outer and inner membranes Mitochondria of this *appearance can be found in cells from the peripheral and central zone as well* OsO_4 fixation × 20 600

The system of canaliculi, which probably represents the endoplasmatic reticulum, is well developed in the basal portion of the cytoplasm, but may be found in the apical (supranuclear) region as well In a small number of sections it was present in cells in the intermediate zone of the tumour islands *Finally small dense granules of about 150 Å in diameter are distributed freely* in the cytoplasm of the peripheral cells



Fig 7
High magnification electron micrograph of attachment plaques of the intermediate cells Between the plaques a layer of low density is seen Bundles of tonofilaments anchor themselves to the internal face of each attachment plaque OsO_4 fixation × 80 000

The cells of the *intermediate zone* are surrounded by a distinct plasma membrane characterized by numerous foldings or interdigitations (Fig 5) Inbetween the plasma membranes at points of close contact a narrow zone of lower electron density can be distinguished (Fig 6 a) The detailed composition of contact zones vary to some extent In some cases there is a slight increase in density and distinctness of the cell membranes whereas in other areas the point of contact is composed of three layers or zones namely two dense ones separated by a layer of lower density In several contact areas there is an accumulation of dense osmiophilic material on the inner aspect of the joining plasma membranes (Figs 6 a and 7) In this way the proper attachment plaques or desmosomes are formed This structure is found to be a very characteristic feature of the intermediate cell layer although attach



Fig 8

Large central cells containing mitochondria (M) and circular profiles (P) of varying size The plasma membranes show a striking degree of convolution The cytoplasmic fingerlike projections interdigitate with similar projections of neighbouring cells Between the cells are irregular clefts At the top of the micrograph a large cavity bordered by microvilli (v) N nucleus OsO_4 fixation × 6 250

ment plaques could be demonstrated at the supranuclear or nuclear level of the columnar as well (Fig 3) Finally desmosomes are present between the central cells which are adjacent to the intermediate cell layer

The cytoplasm of the intermediate cells is often dominated by bundles of fine filaments (Fig 6 a) The bundles are seen running between each other in all directions, in the vicinity of the plasma-membrane they gather into thicker bundles or tufts which seem to terminate or to be embedded in the osmiophilic attachments (Fig 7) Usually, the desmosomes are located on the lateral aspects of the fingerlike cytoplasmic



Fig 9
Part of a central cell showing a vacuole bordered by microvilli (v) The content of the vacuole seems different from the content of the intercellular clefts (I) The superficial parts of the cytoplasm contain many small circular profiles N nucleus OsO_4 fixation × 11,500

projections thus showing an edge toward the cell nuclei (Fig 6 a) As a rule the desmosomes receive the tonofilaments at an acute angle

Mitochondria of typical morphology and size (0 2 0 2) micron) are sparsely distributed throughout the cytoplasm of the intermediate cells (Fig 6 c) In some cells small round profiles are seen in the vicinity of the nucleus (Fig 6 b) in rare cases accompanied by single flattened sacs These structures are considered to represent the *Golgi* apparatus Granules of 150 Å in size are evenly distributed in the cytoplasm of the intermediate cells though usually rather few in number the endoplasmic reticulum is but little developed or not present at all

The cytoplasm of the *central cells* contains few mitochondria they



Fig 10

Central cells of a tumour island M mitochondria v microvilli Granular or vesicular masses presumably originating from disintegrating cells are found in the intercellular spaces OsO_4 fixation $\times$ 6 050

are of the same type as those encountered in the peripheral and intermediate cells, but some show signs of degeneration Attachment plaques or desmosomes and bundles of tonofilaments occur seldom in the most centrally placed cells in the tumour island, but are as previously indicated seen at the junction between central and intermediate cells A characteristic cytoplasmic component is found in the central cells, these are small circular profiles of varying sizes usually not exceeding 0 1 micron in diameter, localized close to the plasma membrane The plasma membranes of the cells in question usually follow a very complicated course and have irregular processes, folds, and invaginations (Fig 8) The cell-membranes are in some areas closely approximated while in other places irregular clefts separate the individual cells Cells

taking part in the limitation of large intercellular spaces are often provided with fingerlike projections or microvilli (Figs 8 and 10) Vacuoles without obvious mutual connections with intercellular clefts can be clearly seen in these cells (Figs 2 an 9) The limiting membrane of these vacuoles are likewise provided with microvilli The intercellular clefts may contain material possibly originating from disintegrating cells (Fig 10)

The nuclei of the different cell types in the ameloblastoma vary in morphology While in the peripheral cells the nuclear shape is often oval (Fig 3), those of the intermediate and central layers are invariably round (Figs 5 and 8) The triple-layered nuclear membrane shows now and again deep invaginations The internal structure of the nuclei is composed of a rather fine granular substance and in quite a number of nuclei one or several nucleoli can be seen (Figs 2 and 3)

DISCUSSION

In the greater part of the ameloblastoma examined three cell zones can be distinguished a peripheral, an intermediate, and a central zone Where adjacent zones meet, transitional celltypes are seen and it is natural to assume that the central polyhedrical cells are derived from the intermediate stellate cells and that the latter in their turn are derived from the peripheral cells

The peripheral cells of the tumourous islands resemble the cells of the inner enamel epithelium of the enamel organ, a fact emphasized by several authors (cf *Bernier* 1955 and *Langer* 1958)

The present study shows that the ultrastructure of the peripheral cells varies some of the cells are characterized by infra or supranuclear accumulation of *mitochondria* others by the presence of irregular cavities or vacuoles containing a substance of unknown composition, fine bundles of filaments are seen in the cytoplasm of these cells

This variation in ultrastructure might be interpreted as differences in degree of differentiation and it would be of considerable interest to compare the ultrastructure of the peripheral cells of the simple ameloblastoma with the ultrastructure of the inner enamel epithelium at different stages of differentiation However, the ultrastructure of the inner enamel epithelium in man are not described to any extent in the articles hitherto published on electronmicroscope studies of amelogenesis Furthermore, most of the work published is concerned with the more advanced stages of amelogenesis (*Lenz* 1957, 1958, 1959, and *Nylen & Scott* 1958 a) There seem, however, judging from the published photomicrographs to exist distinct points of resemblance between the ultrastructure of the inner enamel epithelium cells and the [illegible]
cells of th[illegible]

only a slightly developed *Golgi*-apparatus, probably indicating a rather low metabolic activity. The most characteristic components of the cytoplasm of these cells are tonofilaments and the desmosomes Tonofilaments and desmosomes have been described to occur in many epithelial tissues, as in stratum spinosum of the epidermis (*Selby* 1955, *Charles & Smiddy* 1957, *Odland* 1958, and *Hibbs & Clark* 1959), and in the non-cornified stratified squamous epithelium (ctr *Vogel & Glatthaar* 1958) It is assumed that desmosomes play an important role in the mechanical stability of the tissues In fully differentiated stratified squamous epithelium the number of desmosomes is great, exceeding considerably the number demonstrated in the epithelial tumour presented here

The tonofilaments are coarser and the intercellular spaces smaller in the ameloblastoma as compared with normal or tumorous squamous epithelium Typical desmosomes are regularly seen between the cells of stellate reticulum and between the cells of the stratum intermedium of the enamel organ, at certain stages of matrix-formation they have been demonstrated between the ameloblasts and the cells of the stratum intermedium (*Nylen & Scott* 1959) In the simple ameloblastoma examined we have not observed a cell-layer corresponding to the stratum intermedium of the enamel organ It is believed that the presence of this layer is of importance in the formation of the hard dental tissues (*Marsland* 1951) The problem of the occurrence of this layer in ameloblastomas which does not produce hard dental tissues is not clarified (*Manley* 1954, and *Hodson* 1957)

The large central cells of the ameloblastoma contain few mitochondria as do the intermediate cells The cells are kept in contact with each other through folded or villous interdigitating projections of the plasma-membranes A similar cell type seems not to occur in the normal enamel organ On the other hand the central cells resemble the superficial cells of the stratified squamous epithelium of the oral mucous membrane described by *Sognnaes & Albright* 1958 As a rule the central cells do not show desmosomes or tonofilaments, their microvilli and small vesicles might play a role for the exchange of fluid between the cells and the intercellular spaces which they adjoin Cytoplasmic structures of this kind are known to be present in cells with absorptive and secretory activities

In summarizing it may be said that the outer cell-layers of the ameloblastoma-islands resemble the outer cell-layers of the enamel organ The intermediate layer in the islands have points of similarity with both the stellate reticulum of the enamel organ and stratum spinosum in stratified squamous epithelium Furthermore the central parts of the ameloblastoma-follicle resemble the superficial layers in stratified squamous epithelium Thus the degree of cell-differentiation of the tumour-tissue is similar to that of the cells of the dental lamina at the time of development of this structure However, thorough knowledge of the ultrastructure of the normal odontogenic tissue has to be gained

before the degree of differentiation of the ameloblastoma can be established

SUMMARY

Tissue removed from a simple ameloblastoma of the lower jaw of a 40 year old woman was examined in electron microscope after fixation in buffered OsO_4 and embedding in metacrylate and by light microscopy after routine preparation

Three cell zones can be distinguished in the tumour islands a peripheral cylindrical single-cell layer, an intermediate layer composed of star shaped cells and in the central parts polyhedrical cells some of which may undergo cystic degeneration The star-shaped cells form the bulk of the tumour islands and contain tonofilaments and desmosomes and a few mitochondria The central cells have irregular folded plasma membranes, they are often closely approximated and contain few mitochondria tonofilaments and attachment plaques The cell membrane may show microvilli and in the superficial zones of cytoplasm small vesicles can be found

The ultrastructure of the peripheral cells varies, a number of cells are characterized by irregular cytoplasmic cavities or canals containing a homogenous substance In contrast to the intermediate cells they contain few desmosomes and tonofilaments

The structure of the tumour tissue is compared with that of the enamel organ and stratified squamous epithelium and the degree of differentiation is discussed

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REFERENCES

Bernier J L The Management of Oral Disease p 515-578 C. V. Mosby Company St Louis 1959

Caulfield J B Effects of varying the vehicle for OsO_4 in tissue fixation J biophys biochem Cytol 3 827 829 1957

Charles A & Smiddy F The tonofibrils of the human epidermis J invest dermatol 29 327 338 1957

Dalton A J & M D Felix In Fine Structure of Cells Symp ... 274 293 Noordh ...

Fassk [illegible]

Gorlin R J Chaudhry A P & Pindborg J J The odontogenic tumors—their classification histopathology and clinical behavior in man and domesticated animals Cancer 1961 In press

Hibbs R C & Clark W H Electron microscopic studies of the human epidermis The cell boundaries and topography of the stratum Malpighii J biophys biochem Cytol 6 71 76 1959

Hodson J J Observations on the origin and nature of the adamantinoma with special reference to certain muco epidermoid variations Brit J plast Surg 10 38 59 1957

Horstmann F & Knoop A Elektronenmikroskopische Studien an der Epidermis Z Zellforsch 47 348 362 1958
Langer F Histopathologie der Tumoren der Kiefer und der Mundhöhle p 4 17 Georg Thieme Verlag Stuttgart 1958
Lendrum A C The phloxin tartrazine method as a general histological stain and for the demonstration of inclusion bodies J Path Bact 59 399 404 1947
Lenz H Elektronenmikroskopische Untersuchungen der Ameloblastenschicht Dtsch zahnärztl Z 12 1568 1576 1957
Lenz H Elektronenmikroskopische Untersuchungen der Schmelzgenese Dtsch zahnärztl Z 13 991–1005 1958
Lenz H Bestehen zwischen Schmelz und Dentin praeformierte Bahnen? Elektronen mikroskopische Untersuchungen Dtsch zahnärtl Z 14 272 279 1959
Manley E B Adamantinoma in relation to tooth development Aust J Dent 58 137–150 1954
Marsland E M A histological investigation of amelogenesis in rats I Matrix for mation Brit dent J 91 251 261 1951
Nylen Marie Ussing & Scott D B Electron microscopic studies of the early stages of amelogenesis Abstract J dent Res 37 48 1958 (a)
Nylen Marie Ussing & Scott D B An Electron Microscopic Study of the Early Stages of Dentinogenesis Public Health Service Publication No 613 U S Dept of Health Education and Wellfare Washington D C 1958 (b) 55 pp
Nylen Marie Ussing & Scott D B Personal communication September 9 1959
Odland G F The fine structure of the interrelationship of cells in the human epidermis J biophys biochem Cytol 4 529 538 1958
Palade G F A study of fixation for electron microscopy J exp Med 95 285 298 1952
Pflüger H Über die vom zahnbildenden Gewebe ausgehenden Geschwulste Adamantinom und Odontom Dtsch Zahn Mund u Kieferheilk 27 97 121 1956
Pindborg J J & Clausen F Classification of odontogenic tumors Acta odont scandinav 16 293 301 1958
Selby C C An electron microscopic study of the epidermis of mammalian skin in thin sections J biophys biochem Cytol 1 429 444 1955
Sjöstrand F S A new microtome for ultra thin sectioning for high resolution electron microscopy Experientia 9 114 115 1953
Small T A & Waldron C A Ameloblastomas of the jaws Oral Surg Oral Med Oral Path 8 281 297 1955
Sognnaes R F & Albright J T Electron Microscopy of the epithelial lining of the human oral mucosa Oral Surg Oral Med Oral Path 11 662 673 1958
Thoma K H The pathogenesis of the odontogenic tumors Oral Surg Oral Med Oral Path 11 662 673 1951
Vogel A & Glatthaar E Weitere elektronmikroskopische Untersuchungen am Portiokarcinom Oncologia 11 138 147 1958

FROM THE DEPARTMENT OF PATHOLOGY UNIVERSITY OF UPPSALA SWEDEN

ON THE NATURE OF COBALT INDUCED CHANGES IN THE ALPHA CELLS OF THE ISLETS OF LANGERHANS IN THE GUINEA PIG

By

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Received 22 xii 60

The characteristic change in the α cells observed by *van Campenhout & Cornelis* (2 3 4) after the administration of cobaltous chloride to guinea pigs was interpreted as elective damage to the α cells resulting from the cobaltous chloride Attempts have also been made to explain similar findings in other animals by the same reasoning Observations suggesting other explanations have however been made and divergent opinions have been expressed regarding the changes Experiments applying vital staining in rats rabbits and guinea pigs treated with cobaltous chloride did not for instance confirm the previous assumption that this substance may cause damage to the α cells (17) Nor has examination of the pancreas of rats by electron microscopy after the administration of cobaltous chloride revealed changes comparable to the β cell damage induced by alloxan (25) *Lacy & Cardeza* (19) likewise observed no changes in the nuclei of the α cells of guinea pigs but noticed reversible degranulation as well as vacuolisation of the cytoplasm and reversible changes of the mitochondria in some α cells

The nature of vacuolisation of the α cells in the guinea pig is not yet clear and the object of the present investigation was to endeavour to throw light on this problem

MATERIAL AND METHODS

Guinea pigs (both males and females) received daily for 4 days a subcutaneous injection of cobaltous chloride (20–30 mg/kg) or cobaltous nitrate (30–40 mg kg) and were then killed together with control animals that had received the solvent (0 9 per cent NaCl solution) in the same ...

... us (12 14) Even if it a priori seemed

Supported by grants from *Magnus Bergvalls Stiftelse* Stockholm

unlikely that changes in the islet volume could arise within the short period during which the experiment was in progress a calculation of the area of the islets in relation to that of the pancreas was made by tracing off the pancreas surface at a magnification of 12 and the islet surface at an approximate magnification of 200 and determining the area by planimetry

RESULTS

After the administration of cobaltous chloride a high degree of vacuolisation was observed in nearly 30 per cent of the α-cells in all the fixations used (Fig 1, 2) Variations in degree were, however, apparent for the different fixations, the changes being most marked in the specimens fixed in formalin (Fig 1 a) and least noticeable, although quite distinct, in the unfixed, frozen preparations (Fig 1 c)

Tables 1 to 3 show data obtained at the quantitative examination of the islet cells

The karyometric determination (Table 1) revealed an increase of nuclear size in the α-cells of the guinea pigs both after the administration of cobaltous chloride and after cobaltous nitrate The β-cells, on the other hand, showed a slight decrease in the nuclear size although, in the present material, it could not be statistically established

In a pregnant animal treated with cobaltous chloride but not included in the series in Table 1, the nuclear size was somewhat increased in the α cells (30 9 μ^2 as compared with 29 2 μ^2 in the controls), and was approximately the same in the β-cells (26 4 μ^2) as the corresponding figure in the controls (26 8 μ^2) Vacuolisated α-cells were observed, although in lesser numbers than in the other animals (6 5 per cent as against 27 6 per cent)

A comparison of the vacuolisated and non-vacuolisated α-cells with respect to nuclear size showed in most instances a higher mean value for the former group (35 1 μ^2 as against 32 2 μ^2) In Fig 3 will be seen the frequency curve for nuclei in vacuolisated and non-vacuolisated α-cells in the animals given cobaltous chloride in relation to the values for the control animals the α cells in the experimental animals display a broader nuclear size spectrum and a displacement in the direction of higher values with respect to nuclear size Compression with deformation of the nucleus, as well as fibres and septa of cytoplasm between the nucleus and the cell membrane, were sometimes observed in the vacuolated α cells (Fig 2) The nuclear structure was also clearly distinguishable in these deformed nuclei and nuclear pyknosis was seen only in one or two isolated cases (Fig 2 b)

Fig 2 a and b

α cells in an islet of Langerhans in a guinea pig after administration of cobaltous nitrate showing vacuoles and some nuclear deformation but with retained nuclear structure (a) Pyknosis distinguishable in only one cell (b) Bouin s fluid Paraldehydefuchsin Ponceaufuchsin a) × 2200 b) × 1400

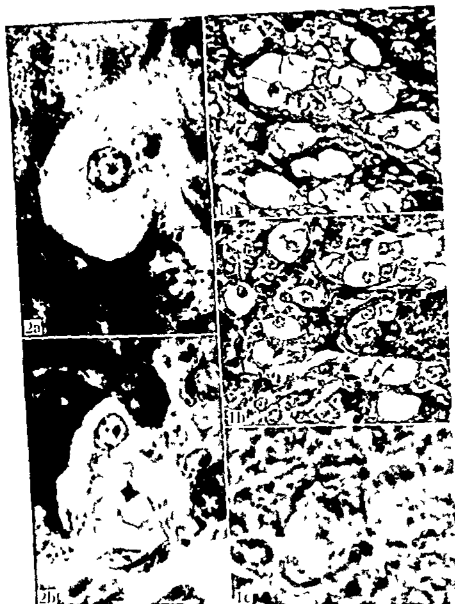


Fig 1

Guinea pig pancreas with vacuolisated α-cells after administration of cobalt salt
Paraldehydefuchsin Ponceaufuchsin

a) Formalin fix × 500
b) Romeis fix × 500
c) Frozen sections of unfixed pancreas with two vacuolised cells
Phase contrast × 2200

TABLE 1

Nuclear Size in α- and β-Cells (μ²) and β/α Value

	α cell nuclei mean value	β cell nuclei mean value	β/α
Control animals	29 2	26 8	0 911
Animals injected with cobaltous chloride	32 3	25 5	0 791
df	7	7	7
t	2 088	1 12	3 21**
Control animals	31 5	31 0	0 989
Animals injected with cobaltous nitrate	38 6	28 8	0 746
df	3	3	3
t	8 961**–***	1 347	2 818

TABLE 2

Number of α Cells in per Cent of Total Number of Islet Cells

	Number of α cells total number of islet cells mean value %	Highest and lowest values
Control animals	11 6	12 4 10 7
Animals injected with cobaltous chloride	17 2	17 4 17 0
df	6	
t	12 57***	

TABLE 3

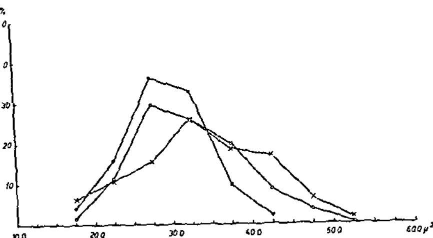
Area of Pancreas Islets in per Cent of Total Pancreas Area

	Islet area pancreas area mean value %	Highest and lowest values
Control animals	1 55	1 83 1 25
Animals injected with cobaltous chloride	1 97	2 14 – 1 85
df	6	
t	2 69*	

df degrees of freedom
t Student's t test
* probable
** very probable
*** significant
§ nearly probable

The differential count of the islet cells (Table 2) revealed a definite increase, by nearly 50 per cent, in the relative frequency of α cells

An increase by 25 30 per cent in the islet surface in per cent of the total pancreas surface was demonstrated (Table 3)



F g 3

Frequency curve f r α-cell nuclei

—— α-cell nuclei in control animals
x——x α-cell nuclei in vacuolisated cells after administration of cobaltous chloride
o——o α cell nuclei n n n vacuolisated cells after administration of cobaltous chloride

DISCUSSION

The investigation confirmed the observations of van Campenhout and Cornelis regarding vacuolisation of the α cells after the administration of cobalt salt A study of islet cells hardened in different fluids and especially of the cells in frozen sections of unfixed material suggested that the vacuolisation of the α cells is not a fixation artifact However as the vacuolisation was more marked in formalin hardened specimens than in those fixed by other techniques a certain element of artifact is probably present *Falkmer* (8 9) made a similar observation in the α cells of certain bony fishes changes in the α cells could be demonstrated with the aid of some formalin fixation methods but not with others and therefore should probably be interpreted as an artificial product

As was the case in an earlier work (17) this investigation as well revealed no definite destruction of the α cells A compressed deformed nucleus with the nuclear structure retained was seen in some of the highly vacuolisated α-cells as van Campenhout and Cornelis have already reported *Creutzfeldt & Schmidt* (6) who only occasionally observed nuclear destruction when counting the islet cells considered that the α cell changes are not a manifestation of primary damage but instead arise as a result of abnormal stress The changes in nuclear size suggest that there is increased activity in the α cells after the administration of cobalt salts and that this heightening of activity has reached a maximum in some of the vacuolisated cells The nuclear compression observed in some of the vacuolated α-cells may probably

be interpreted as reflecting a maximum accumulation in the cytoplasm and distention of the cell membrane by secretion products, perhaps as a termination of the cycle of activity The differential count and volume determination also suggest that the α-cells are subject to a progressive transformation similar to that observed in rabbits after the administration of cobaltous chloride over a period of 10 to 11 days (11) Cobalt salts appear to have an α-cytotropic rather than an α cytotoxic effect Thus, morphologically speaking, we have here an analogy with the β-cytotropic action of the hypoglycaemic sulphonamides (21) It is not yet clear whether the analogy also applies to the function

At its present stage the investigation has provided no definite clue to the primary cause of the heightened activity in the α-cells Several possibilities arise to the mind, namely, a direct, stimulating effect on the α cells by cobalt salts, an indirect effect on the α-cells as a result of a metabolic, hormonal, or enzymatic disturbance caused by the action of cobalt salts on other organs or organic systems, or a neurogenic (sympathetic system) stimulation of the α-cells directly or indirectly produced by the cobalt salts Of these alternatives, the last two would appear to be more likely than the first Among other things, it has not been demonstrated that Co^{60} becomes localized to the islets of Langerhans (28) The fact that knowledge is still insufficient regarding the changes occurring in the blood sugar after the administration of cobalt salt to the guinea pig makes it difficult to go more deeply into the question of whether the mechanism has any connection with a metabolic disturbance *Van Campenhout & Cornelis* (3, 4) noted hyperglycaemia in this animal after intracardial administration of cobalt salts but not after repeated subcutaneous injections In rats, cobalt salt administration results in a considerable degree of hyperglycaemia with the maximum about 2 hours after subcutaneous injection (15, 16), and it seems likely that similar conditions should exist for guinea pigs From the evidence of experiments at present in progress this would also appear to be the case, with a single subcutaneous injection in guinea pigs It is, however, difficult to believe that hyperglycaemia could be the cause of the α-cell change, on the contrary, one would almost expect to find signs of diminished α cell activity in association with hyperglycaemia It would seem logical to consider the possibility that hyperglycaemia might be a sequel of hyperactivity of the α-cells This presumption is contradicted, however, by the observation that hyperglycaemia can also be produced in pancreatectomized or partially eviscerated dogs (20) Other metabolic disturbances besides hyperglycaemia may occur after administration of cobalt salts, in proteins, for instance (27, 22) as well as in lipoprotein and cholesterol (5, 22, 24, 1) and in glutathione (13), but it seems improbable that there could be any direct causative connection between these changes and the α-cell change As regards hormonal factors, the opinion has been advanced that the cobalt effect probably cannot be produced in pregnant ani

mals The findings in one of our cases belie this assumption A resistance to cobalt action in young guinea pigs of a few days old is also noted (18)

Several observations suggesting a connection between the adrenals and sympathetic system and the cobalt salt effect have been made It should be especially stressed that the effect of cobalt salt on the blood sugar is not observed after an adrenalectomy (10, 15) or after the administration of dihydroergotamine (7, 1) Sympatheticsystem adrenalin stimulation with glycogenolysis in the liver also demonstrated histologically (23), would be an acceptable explanation of the mechanism in hyperglycaemia

It is not known whether the characteristic change in the α cells in guinea pigs can also be attributed to a similar mechanism but it seems likely It is interesting to note that in cats the α cells increase in number after vagotomy, sympathetic stimulation, or an adrenalin injection (26) If the α cell change is attributable to a sympathetic system-adrenalin effect the question arises whether the effect is transferred to the α cells by nervous pathways or by humoral transmission

SUMMARY

Examination of the cells in Langerhans islets with the aid of karyometry and a differential count revealed a relative increase in the number of α cells as well as increase of nuclear size in the α cells after subcutaneous injections of cobaltous chloride and cobaltous nitrate over a period of a few days The islets also displayed a slightly increased relative volume in relation to the exocrine pancreatic parenchyma The characteristic α cell change, with vacuolisation of some of the cells, has therefore been interpreted as a phenomenon of a progressive nature (α cytotropic effect) Its genesis is discussed It is suggested that the change may be a result of heightened activity in the adrenal and/or sympathetic system induced by cobalt salts

REFERENCES

1 Boyd G S & MacLean N Observations on the metal [illegible]

2 [illegible]

3 [illegible]

4 van Campenhout F & Cornelis G Destruction expérimentale des cellules alpha des îlots endocrines du pancréas Bull Acad Royal Méd Belg ser 6 16 382 400 1951

5 Caren R & Carbo L Pancreatic alpha [illegible] metabolism J clin E[illegible]

6 Creutzfeldt W & Schmidt W [illegible] Blutzucker und die Lang[illegible] exp Path u Pharm 222 [illegible]

7 *Ellis S Anderson H I & Collins M C* Pharmacologic Differentiation Between Epinephrine- and HGI Hyperglycemias Application in Analysis of Cobalt Hyperglycemia Proc Soc Exp Biol Med *84* 382-386 1953

8 *Falkmer S* Histological histochemical and electronmicroscopical investigations on the cells of the endocrine pancreatic tissue of the marine teleost *Cottus scorpius* Kungl Fysiograf Sallsk Handl N F *71* 1960 (in press)

9 *Falkmer S* Marine Teleosts as Experimental Animals in Diabetes Research Acta path et microbiol scandinav Suppl 144 vol 51 1961

10 *Franck C Lamarche M & Kocaret R* *Sur le mécanisme de l'hyperglycémie* précoce provoquée chez le cobaye par administration de chlorure de cobalt Compt rend Acad Sci *245* 1165 1167 1957

11 *i Holt C & L* Die Wirkung von Kobalt and Cadmium auf die A Zellen der Langerhanschen Inseln Z f Naturforsch *9 b* 319 325 1954

12 *Hultquist G T Dahlen M & Helander C G* Über die Technik bei Darstellung und Zählung der sogen Silberzellen in den Langerhans schen Inseln Schweiz Zschr f allg Path u Bakt *11* 570 589 1948

13 *Hultquist G* The effect of cobalt chloride and synthaline A on reduced glutathione in blood and tissue in rats with partial pancreatic duct ligation Brit J Exp Path 37 357 360 1956

14 *Hultquist G T* Effect of certain sulfhydryl blocking substances on blood sugar and islets of Langerhans in rats Acta path et microbiol scandinav 4[illegible] 153 159 1959

15 *Hultquist G* Om mekanismen för koboltkloridverkan på råttor Nord Med *62* 1686 1687 1959

16 *Hultquist G T* Effect of Cobaltous Chloride on the Blood Sugar Level and the Islet Cells in Rats Experientia *15* 340 1959

17 *Hultquist G T* Effect of Cobaltous Chloride Synthaline A and IPTD on the Islets of Langerhans Studied by Means of Vital Staining Acta Soc Med Upsal *64* 255 260 1959

18 *Korp W & LeComple P M* The Nature and Function of the Alpha Cells of the Pancreas Diabetes *4* 347 366 1955

19 *Lacy P E & Cardeza A F* Electron microscopy of Guinea pig pancreas Effect of cobalt on the acini and islets Diabetes 7 368-374 1958

20 *Lazarus S S Goldner M G & Volk B W* Selective destruction of the pancreatic alpha cells by cobaltous chloride in the dog Metabolism *2* 513 520 1953

21 *Loubatières A* Analyse de l'action beta cytotrope des sulfamides hypoglycémiants Presse Med *68* 1421 1424 1960

22 *Marrone N Vannini P & Barbara I* Studio sugli effetti bioumorali e istol

23 *Marrone N Barbara I & Vannini P* Studio sugli effetti bioumorali e istologici del cloruro di cobalto nel conigli II Osservazioni istologiche e istochimiche sulla ghiandola surrenale sul fegato e sulla tiroide Arch Sc Biol *43* 231 244 1959

24 *Mukherjee S K De I N & Mukerji B* Alpha Cell Activity and Its Relation *to Glycaemic Level and Insulin Sensitivity in Albino Rats* Indian J Med Res *45* 337 344 1957

25 *Nordmann M & Wolf F* Elektronenoptische Untersuchungen des Pankreas unter normalen und abnormen Stoffwechsellagen Virch Arch f path Anat *333* 54 67 1960

26 *Sergeyeva M A* Microscopic changes in the islands of Langerhans produced by sympathetic and parasympathetic stimulation in the cat Anat Rec 77 297 317 1940

27 *Simon A Arnould P & Franck C* Electrophoresis of serum proteins in the course of *hyperglobulinaemia produced by cobalt chloride in the guinea* pig Compt rend Soc Biol Paris *152* 997 998 1958

28 Ulrich F & *Copp D H* The metabolism of radioactive cobalt (Co[60]) in normal and alloxan diabetic rats Arch Biochem *31* 148 153 1954

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IMMUNOCHEMICAL STUDIES ON SOME SEROLOGICAL CROSS-REACTIONS IN THE KLEBSIELLA GROUP

4. *Immunisation of Mice with Purified Capsular Polysaccharide*

By

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The antigenicity of purified pneumococcal capsular polysaccharide in mice and man has been well established, likewise the long lasting protective value in both species of small doses of polysaccharides (2, 4, 5, 6, 7, 8, 11, 12).

Felton & Ottinger (3, 4) showed that large doses of types 1 and 2 pneumococcal polysaccharide caused an apparently life-long specific immunological paralysis.

The experiments reported in this paper were carried out to study the behaviour of some Klebsiella capsular polysaccharides under similar experimental conditions.

METHODS

The capsular polysaccharides were prepared from strains M4-146, a virulent strain of Kl. pneumoniae type 1(A), strain S 57 of Kl. ozaenae [illegible] strain of [illegible]

ho [illegible]
(9 [illegible] the method de-
sc[illegible] the preceding paper (9b), consisting in extraction with cold water, removal of organisms by high speed centrifugation and/or filtrations and precipitation with ethanol acetate.

Mice were [illegible]

[illegible] the LD_{50} in each experi-
[illegible] calculated by the method of *Reed & Muench* (10). The number of organisms corresponding to each LD_{50} was also calculated from the colony counts.

TABLE 1

Immunisation of Mice with Klebsiella Type 1 Capsular Polysaccharide (Doses of 5 µg and and 1000 µg) and Challenge 1 Week Later with the Type 1 strain MA 138

Dilution of culture	5 µg polysaccharide					1000 µg polysaccharide					Controls				
	Within group		Total		Per cent dead	Within group		Total		Per cent dead	Within group		Total		Per cent dead
	D	S	D	S		D	S	D	S		D	S	D	S	
10^{-3}	8	0	13	0	100										
10^{-4}	5	3	5	3	63	7	1	18	1	95					
10^{-5}	0	8	0	11	0	6	2	11	3	79	8	0	20	0	100
10^{-6}	0	8	0	19	0	5	3	5	6	46	5	3	12	3	80
10^{-7}	0	8	0	27	0	0	8	0	14	0	7	1	7	4	64
10^{-8}						0	8	0	22	0	0	8	0	12	0
LD_{50}					$10^{-4.}$[illegible]					$10^{-5.88}$					$10^{-7.}$[illegible]

D dead within 4 days S survived
Bacterial count in culture used for challenge 13×10^{7} per 0.5 ml

TABLE 2

Immunisation of Mice with Klebsiella Type 1 Capsular Polysaccharide (Doses of 5 µg and and 1000 µg) and Challenge 2 Weeks Later with the Type 1 strain MA 148

Dilution of culture	5 µg polysaccharide					1000 µg polysaccharide					Controls				
	Within group		Total		Per cent dead	Within group		Total		Per cent dead	Within group		Total		Per cent dead
	D	S	D	S		D	S	D	S		D	S	D	S	
10^{-3}	8	0	11	18	93	8	0	24	0	100					
10^{-4}	3	5	3	6	33	5	3	16	3	84					
10^{-5}	0	8	0	14	0	4	4	11	7	61	8	0	18	0	100
10^{-6}	0	8	0	22	0	7	1	7	8	47	4	4	10	4	71
10^{-7}						0	8	0	16	0	0	8	6	12	33
10^{-8}											6	2	6	14	30
LD_{50}					$10^{-3.71}$					$10^{-5.70}$					$10^{-6.50}$

D dead within 4 days S survived
Bacterial count in culture used for challenge 30×10^7 per 0.5 ml
§ one of the mice injected with the dilution 10^{-2} survived

TABLE 3

Immunisation of Mice with Klebsiella Type 1 Capsular Polysaccharide (Doses of 5 μg and and 1000 μg) and Challenge 3 Weeks Later with the Type 1 strain MA 148

Dilution of culture	5 μg polysaccharide					1000 μg polysaccharide					Controls				
	Within group		Total		Per cent dead	Within group		Total		Per cent dead	Within group		Total		Per cent dead
	D	S	D	S		D	S	D	S		D	S	D	S	
10^{3}	7	1	8	1	89	7	1	10	1	91					
10^{4}	0	8	1	9	10	1	7	3	8	27					
10^{5}	0	8	1	17	6	2	6	2	14	13	8	0	25	0	100
10^{6}	1	7	1	24	4	0	8	0	22	0	8	0	17	0	100
10^{7}	0	8	0	32	0	0	8	0	30	0	8	0	9	0	100
10^{8}											0	8	1	8	11
											1	7	1	15	7
LD_{50}					$10^{3.50}$					$10^{3.64}$					$10^{6.78}$

D dead within 4 days S survived
Bacterial count in culture used for challenge 19 × 10^7 per 0 5 ml

TABLE 4

Immunisation of Mice with Klebsiella Type 1 Capsular Polysaccharide (Doses of 5 µg and and 1000 µg) and Challenge 4 Weeks Later with the Type 1 strain MA 148

Dilution of culture	5 µg polysaccharide					1000 µg polysaccharide					Controls				
	Within group		Total		Per cent dead	Within group		Total		Per cent dead	Within group		Total		Per cent dead
	D	S	D	S		D	S	D	S		D	S	D	S	
10^{-2}	7	1	10	1	91	8	0	15	0	100					
10^{-3}	3	5	3	6	33	5	3	7	3	70					
10^{-4}	0	8	0	14	0	2	6	2	9	18	8	0	17	0	100
10^{-5}	0	8	0	22	0	0	8	0	17	0	8	0	9	0	100
10^{-6}											1	7	1	7	13
10^{-7}											0	8	0	15	0
LD_{50}					$10^{-2.71}$					$10^{-3.3}$[illegible]					$10^{-5.57}$

D dead within 4 days S survived
Bacterial count in culture used for challenge 13×10^{8} per 0.5 ml

TABLE 5

Immunisation of Mice with Klebsiella Type 1 Capsular Polysaccharide (Doses of 5 µg and and 1000 µg) and Challenge 3 Weeks Later with the Type 1 strain MA 148

Dilution of culture	5 µg polysaccharide					1000 µg polysaccharide					Controls				
	Within group		Total		Per cent dead	Within group		Total		Per cent dead	Within group		Total		Per cent dead
	D	S	D	S		D	S	D	S		D	S	D	S	
10^{-2}	8	0	12	0	100	8	0	15	0	100					
10^{-3}	4	4	4	4	50	4	4	7	4	64					
10^{-4}	0	8	0	12	0	3	5	3	9	25	8	0	30	0	100
10^{-5}						0	8	0	17	0	8	0	22	0	100
10^{-6}											8	0	14	0	100
10^{-7}											5	3	6	3	67
10^{-8}											1	7	1	10	9
LD_{50}					$10^{-3.0}$					$10^{-3.6}$					$10^{-7.2}$

D dead within 4 days S survived

Bacterial count in culture used for challenge 16×10^7 per 0.5 ml

The polysaccharides used in this and the following experiments were prepared by the cold water extraction method, in contrast to those presented in table 1 4

TABLE 6

Immunisation of Mice with Klebsiella Type 1 Capsular Polysaccharide (Doses of 5 µg and and 1000 µg) and Challenge 6 Weeks Later with the Type 1 strain MA 148

Dilution of culture	5 µg polysaccharide					1000 µg polysaccharide					Controls				
	Within group		Total		Per cent dead	Within group		Total		Per cent dead	Within group		Total		Per cent dead
	D	S	D	S		D	S	D	S		D	S	D	S	
10^{2}	8	0	19	0	100	8	0	20	0	100					
10^{3}	3	5	11	5	69	7	1	12	1	92					
10^{4}	6	2	8	7	53	3	5	5	6	45					
10^{5}	1	7	2	14	13	2	6	2	12	14	8	0	20	0	100
10^{6}	1	7	1	21	5	0	8	0	20	0	6	2	21	2	91
10^{7}						0	8	0	28	0	8	0	15	2	88
10^{8}											7	1	7	3	70
											0	8	0	11	0
LD_{50}					$10^{4.07}$					$10^{3.80}$					$10^{7.[illegible]0}$

D dead within 4 days S survived
Bacterial count of culture used for challenge 15×10^{7} per 0.5 ml

TABLE 7

Summary of Results of Immunisation of Mice with two Different Doses of Klebsiella Type 1 Polysaccharide and Challenge with a Type 1 Culture after Different Time Intervals

Interval between immunisation and challenge	Immunised with 5 µg polysaccharide			Immunised with 1000 µg polysaccharide			Controls	
	LD_{50} calculated as		Ratio of LD_{50} to that of controls	LD_{50} calculated as		Ratio of LD_{50} to that of controls	LD_{50} calculated as	
	Dilution of culture	Number of bacteria		Dilution of culture	Number of bacteria		Dilution of culture	Number of bacteria
1 week	$10^{-4.[illegible]}$	8190	1047	$10^{-7.99}$	171	48	$10^{-7.22}$	7.8
2 weeks§				$10^{-4.57}$	7540	216	$10^{-6.90}$	15
2 weeks	$10^{-4.71}$	58500	708	$10^{-5.79}$	487	5.9	$10^{-6.5[illegible]}$	83
3 weeks	$10^{-3.50}$	61090	1148	$10^{-3.64}$	43530	832	$10^{-6.56}$	52
3 weeks†	$10^{-3.00}$	160000	19500	$10^{-3.36}$	69840	8511	$10^{-7.29}$	8.2
4 weeks	$10^{-2.[illegible]}$	253400	771	$10^{-3.[illegible]}$	52960	151	$10^{-5.57}$	350
6 weeks†	$10^{-4.07}$	12760	1660	$10^{-3.8[illegible]}$	19320	2312	$10^{-7.29}$	7.7

§ this experiment has not been shown in the preceding tables

† the polysaccharide used in these experiments were prepared by the cold water extraction method described in a previous paper (9 b)

RESULTS

Experiment No 1 Of three groups of 48 mice each, one was injected with 5 μg doses of type 1 polysaccharide, one with 1000 μg, and the third with none The mice were challenged after one week, with the results shown in Table 1 Both doses of polysaccharide give some protection, and there is no sign of immunological paralysis, but the larger dose seems to give less protection It was thought that this might be due to a delay of the immune response, due to overloading with antigen, and new experiments were carried out with increased intervals between immunisation and challenge

Experiment No 2 Same plan as in No 1, except that the interval between immunisation and challenge was 2 weeks The results, shown in Table 2, are practically the same as in exp No 1, *viz* a more efficient protection caused by the smaller than by the larger dose

Experiment No 3 Same plan, 3 weeks interval The results are shown in Table 3, and indicate that now the two doses of polysaccharide give about equally good protection, about 3 log units, suggesting that at this time the mice which received the larger dose had caught up with those receiving the smaller dose

Experiment No 4 Same plan, interval 4 weeks The results in Table 4 are comparable to those obtained in exp No 3 Both antigen doses give reasonably good protection, c 2 8 and 2 2 log-units respectively

Experiment No 5 In this and the following experiments the polysaccharide was prepared by a different method The interval between immunisation and challenge was 3 weeks Again the polysaccharide gives appreciable protection, with a slight difference in favor of the smaller dose In this experiment the protection produced reached a maximum of more than 4 log units with the smaller dose and nearly 4 log units with the larger dose

Experiment No 6 Same plan, interval 6 weeks The results shown in Table 6 show that in this single instance the larger dose appears to give better protection than the smaller one, but the difference from the other experiments may not be significant

A summary of the results obtained in these experiments is presented in Table 7 It is quite clear that the polysaccharide injections produce some immunity and give the mice a moderate degree of protection, which raises the LD_{50} from 6 fold to nearly 20000-fold in different experiments The results as expected, indicate a wide range of variation, and additional conclusions can be drawn only with great caution It seems however, that the smaller dose of polysaccharide has a better effect in all experiments except the one with the longest incubation time (6 weeks) This may mean that the larger dose causes a certain delay in the development of the level of immunity, possibly by neutralising some of the antibody On the other hand there is no sign of real immunological paralysis

TABLE 8

Immunisation of Groups of Mice with Klebsiella Capsular Polysaccharides of Types 1 (5 µg) 4F (1000 µg) and 21 (1000 µg) and Challenge 4 Weeks later with the Type 1 Strain MA 148

Dilution of culture	Type 1 polysaccharide					Type 4F polysaccharide					Type 21 polysaccharide					Controls				
	Within group		Total		Per cent dead	Within group		Total		Per cent dead	Within group		Total		Per cent dead	Within group		Total		Per cent dead
	D	S	D	S		D	S	D	S		D	S	D	S		D	S	D	S	
10^{-2}	8	0	21	0	100	8	0	32	0	100										
10^{-3}	7	1	13	1	93	8	0	24	0	100	8	0	26	0	100	8	0	34	0	100
10^{-4}	5	3	6	4	60	7	1	16	1	94	8	0	18	0	100	8	0	26	0	100
10^{-5}	0	8	1	12	8	7	1	9	2	82	6	2	10	2	83	8	0	18	0	100
10^{-6}	1	7	1	19	5	0	8	2	10	17	2	6	4	8	33	2	6	10	6	62
10^{-7}	0	8	0	27	0	1	7	2	17	11	2	6	2	14	13	4	4	8	10	44
10^{-8}						1	7	1	24	4	0	8	0	22	0	4	4	4	14	22
10^{-9}						0	8	0	32	0						0	8	0	22	0
LD_{50}					$10^{-3.1}$[illegible]					$10^{-5.19}$					10^{-}[illegible]					$10^{-6.67}$

D dead within 4 days S survived

Bacterial count in culture used for challenge 33×10^{7} per 0.5 ml

It was not practicable to test higher antigen doses than 1000 µg since more concentrated solutions than 1 500 became too viscous to handle

Polysaccharides produced by both methods are effective antigens + The figures suggest that the cold water extraction method may give the more efficient antigen but this may not be so and additional experiments wold be required to show whether the difference is real or not

Experiment No 7 The purpose of this experiment was to find out whether injection of a capsular polysaccharide prepared from the sero type AF which cross reacts with type 1 would protect mice against type 1 infection (9 a 9 b)

In the first attempt two groups of mice were immunised with type 1 (5 µg) and type AF (1000 µg) polysaccharide respectively But when the mice were challenged 3 weeks later with a type 1 culture a satisfactory end point was not obtained in the group receiving type AF polysaccharide and the LD , could not be determined The experiment was therefore repeated and this time a third group of mice were injected with a capsular polysaccharide prepared from Klebsiella type 21 which does not cross react with either type 1 or type AF This group was intended to serve as a control that the injection of capsular polysaccharides did not cause any nonspecific protection The results are shown in Table 8 The mice injected with type 1 polysaccharide show a moderate immunity with an about 300 fold increase of the LD_{50} where as the groups injected with type AF and type 21 polysaccharides show little or no immunity with increases of the LD_{50} (14 fold and 10 fold respectively) which may very well be within the experimental error of the method Although it is possible that there may be a real difference it seems safe to conclude that the type AF polysaccharide dose not cause a specific immunity of sufficient magnitude to be of any value as a protection against type 1 infection It seems that the cross reactivity between these two types is insufficient to cause significant cross protection such as was found in the classical experiments on the cross reaction between Klebsiella type B and pneumococcus type 2 (1)

COMMENT

The results show that purified capsular polysaccharides prepared from Kelbsiella pneumoniae type 1 like pneumococcus polysaccharides are antigenic to mice Although the presence of polysaccharide protein complexes in the preparations could not be excluded it may be noted that these polysaccharides contained little nitrogen less than 1 per cent and that most of the preparations were prepared by a method involving extraction with hot water which probably would have a marked denaturing effect of any protein present in the extract

The immunity produced is not of a very high order and only gives protection against a moderate number of organisms ranging from c

6 to c 20000 LD_{50} in different experiments In this connection it may be mentioned that attempts to detect precipitating antibody in the blood of some surviving mice by means of a gel-precipitation technique, failed to give any evidence of the presence of such antibodies

The immunity appears to be of a fairly long duration, and is still detectable after 6 weeks, but unlike the results obtained by *Felton & Ottinger* (3) with pneumococcal polysaccharides types 1 and 2 the largest dose that could be given, did not produce immunological paralysis, but apparaently only a reduction of the level of immunity in the early stages This may possibly suggest that these polysaccharides do not remain as long in an active form, as the pneumococcal polysaccharides have been shown to do

The finding that the polysaccharide prepared from type AE did not give rise significant to protective immunity, may not surprise in view of the comparatively weak cross-reaction between the two types

REFERENCES

1 *Avery O T Heidelberger M & Goebel W F* The soluble specific substance of Friedländer's bacillus II Chemical and immunological relationships of Pneumococcus type II and of a strain of Friedländer's bacillus J exper Med *42* 709 725 1925

2 *Avery O T & Goebel W F* Chemoimmunological studies on the soluble substance of pneumococcus I The isolation and properties of the acetyl polysaccharide of pneumococcus type I J exper Med *58* 731 755 1933

3 *Felton L D & Ottinger B* Pneumococcus polysaccharide as a paralyzing agent on the mechanism of immunity in white mice J Bact *43* 94 95 1942

4 *Felton L D* The *significance of antigen in animal tissues* J Immunol *61* 107 117 1949

5 *Felton L D Prescott B Kauffmann G & Ottinger B* Pneumococcal antigenic polysaccharide substances from animal tissues J Immunol *74* 205 213 1955

6 [illegible] Antibody formation [illegible] or their type specific [illegible]

7 [illegible] sistence of antibodies [illegible] lysaccharides J Immunol *65* 535 541 1950

8 *Heidelberger M MacLeod C M & DiLapi M* Antigenic potency in man of the specific polysaccharides of types I and V pneumococcus and their products of alkaline degradation J Immunol *66* 145 149 1951

9a *Eriksen J & S D Henriksen* Immunochemical studies on some serological cross reactions in the klebsiella group 2 Chemical composition of the capsular polysaccharides Acta path et microbiol scandinav *45* 387–391 1959

9b *Henriksen S D & Eriksen J* Immunochemical studies on some serological cross reactions in the klebsiella group III Further studies on the cross reactions between types A (1) E (5) and AE Acta path et microbiol scandinav *51* 259 274 1961

10 *Reed L J & Muench H* A simple method of estimating fifty per cent end point Amer J Hyg *27* 493 497 1938

11 *Zozaya J* Carbohydrates adsorbed on colloids as antigens J exper Med *55* 325 351 1932

12 *Zozaya J & Clark J* Active immunization of mice with the polysaccharides of pneumococci types I II and III J exper Med *57* 21 40 1933

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SEROLOGICAL TYPING OF THE STAPHYLOCOCCI BY MEANS OF GEL-PRECIPITATION REACTIONS[1]

By

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Received 3 xii 60

Due to the capacity of the staphylococci to develop resistance to most of the chemotherapeutics, and to the occurrence of staphylococcal nosocomial hospital infections, increasing attention has been focused on the staphylococcal problem Furthermore combating staphylococcal infections have presented considerable epidemiological problems It cannot yet be definitely stated which sources and routes of infection are most important Neither do we know for certain which are the factors that determine the greater sensitivity of the individual patient to staphylococcal infections, and hardly anything is known of possible differences in the virulence of the staphylococci For elucidation of these problems therefore, it is of the utmost importance to be able to classify the pathogenic staphylococci to be familiar with their antigenic structure and to know which staphylococcal antibodies are available in the organism

In previous epidemiological studies phage typing has played the most important role, but relatively few laboratories maintain the necessary staphylococcal phages and practice this technique The types are not clearly defined transitions from one type to another occur frequently, and a number of the staphylococcal strains cannot be typed by means of this method

Serological typing of the staphylococci has been attempted on numerous occasions The best results are obtained by means of the slide agglutination reactions It is agreed that pathogenic staphylococci may be classified in three serological groups as set forth by [illegible]
(5) [illegible]
cocci [illegible]
of us [illegible]
the r [illegible]
Kraj [illegible] [illegible] have continued

[1] The present studies were carried out with the aid of a grant from *P A Brandt s Foundation*

investigation of serological typing of staphylococci by means of the slide agglutination reactions, and both investigators have been able to use this method in epidemiological studies The widespread occurrence of common antigens among the staphylococci and their extremely complex antigenic structure have, however, necessitated the extensive employment of absorbed sera (*Pillet & Orta* (19, 20, 21)) and the inactivation of some of the antigens by heat, trypsin or alcohol *Julianelle* (10). *Hegemann* (6), *Hobbs* (7) and *Krag Andersen* (1) have tried using the complement fixation reaction, but this method does not give type specific reactions *Julianelle & Wieghard* (11, 12, 13), *Cowan* (5), *Verwey* (22), *Hobbs* (7) and *Hegemann* (6) have attempted to use precipitin reactions, by which it is possible to differentiate between the pathogenic and non-pathogenic staphylococci, but which are not successful in establishing a subdivision of the pathogenic staphylococci

Summarizing, it may be said that of the serological methods used for typing the staphylococci, the slide agglutination reactions have so far proved to be the best By means of this method it is practicable with certainty to divide the pathogenic staphylococci into Cowan's three groups A more extensive classification of the staphylococci is possible, but then the types are not well defined and results cannot always be reproduced

On the basis of the foregoing, the author has previously (8) investigated the gel-precipitation reactions in typing the pathogenic staphylococci This method has an advantage in that it affords a simultaneous differentiation between several different antigens Immune sera produced by immunizing rabbits with formalin-killed staphylococci were employed for the experiments It was found that the great difficulties involved in serological typing of the staphylococci are due to the widespread occurrence of several common antigens (9) It was seen that quantitative differences in the occurrence of these common antigens from one culture to another of the same strain complicate their classification and explain the difficulties encountered in recognizing serological types By means of 5 sera, however, it was possible to divide the staphylococci into 6 definite types These findings have since in part been confirmed by *Cohen* and collaborators (4)

The above-mentioned attempts to classify the staphylococci by means of the gel-precipitation reactions (8) led to the observation that all normal human sera contain antibody against an antigen which is present in most of the pathogenic staphylococci (antigen A) (9) Further investigations show that, besides the antibody against antigen A, a few normal sera contain antibodies against other staphylococcal antigens

The greatest difficulties encountered on previous attempts to use the gel-precipitation reaction for typing the staphylococci have been due to the employment of large amounts of undiluted immune sera which are difficult to produce, as well as to the fact that immune sera obtained by

immunization of experimental animals with staphylococci generally contain many different antibodies and thus are difficult to render specific by means of absorption

These two difficulties may be overcome by using the spontaneously occurring staphylococcal antibodies in human sera for the typing, as these sera can be obtained in large amounts and, furthermore, they have most often been found to contain antibody against only one or, at the most, two antigens other than antigen A

MATERIAL AND METHODS

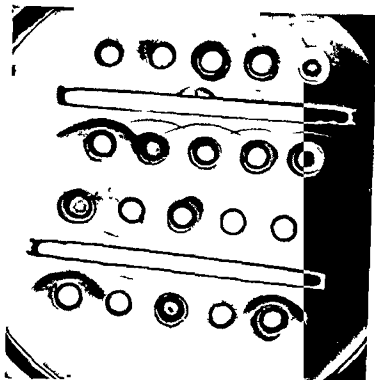
The sera from patients and normal donors used in these investigations were examined by means of the gel precipitation reactions with extract of various staphylococcus strains The staphylococci employed were pure cultures from patients with staphylococcal infections All the strains were coagulase positive The staphylococci were grown on veal infusion agar at 37° C for 14 hours the growth was then scraped from the medium and extracted with isotonic phosphate buffer, with pH approx 6, at room temperature for 14 days, after which the suspension was employed directly for the tests In the present experiment each serum was examined against 20 different staphylococcal strains In order to obtain antibodies against as many different staphylococcal antigens as possible a new batch of staphylococcal strains was employed for every 200 se[illegible]

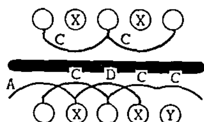
[illegible] freezing of the plasma produces flocculation and lessens the antibody content of the citrated plasma

RESULTS

So far examination has been made of sera from 951 donors, each serum being tested against 20 staphylococcal strains All these sera contained antibody against antigen A, while 240 of the sera contained antibody against one or several other antigens in the staphylococcal strains employed Most of these sera, however, contained only small amounts of antibody and therefore could not be used Furthermore, antibody against the same antigen was frequently found in several of the sera Of the above-mentioned 240 sera, 19 were selected for more detailed examination So far, however, only seven of these sera have proved to be serviceable for further investigations

These seven sera contain antibodies against eight different staphylococcal antigens As far as possible, only sera containing antibodies against one or two antigens were chosen, but nevertheless it was found that these sera were not specific enough to allow typing without employment of special methods for identification of the antigens This can be seen most distinctly in Fig 1 In this experiment the cups marked A were filled with a staphylococcus strain which is known to contain antigen C The remaining cups were filled with various unknown strains





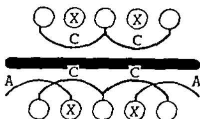
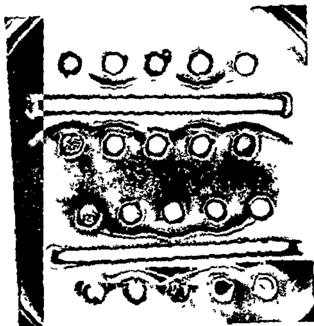


Fig 1
Gel precipitation reaction between serum from a healthy donor (in the long wells) and various staphylococcal strains (in the cups) The cups marked X contain the antigen C for which the unknown staphylococci in the other cups are to be examined Note the reaction of non identity between bands D and C and A and C and the the reaction of identity between the band in front of cup Y and band C

which were to be examined for their possible content of antigen C It will be seen that the serum in the long wells contains antibody against antigen C, which causes the precipitation bands marked C In addition, this serum contains two other antibodies namely D which causes the precipitation band marked D, and A which causes the precipitation bands marked A Without a comparative strain in the cups marked X it would not have been discovered that the D band was caused by an antigen (D) other than the one (antigen C) for which the unknown



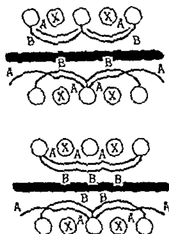
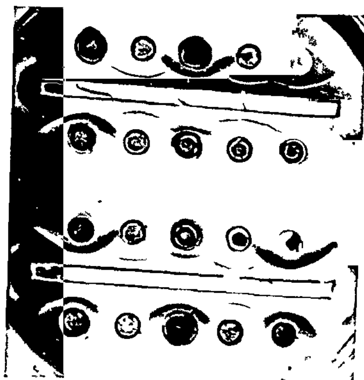


Fig 2
Same experiment as illustrated in Fig 1 but with another donor serum containing antibodies against antigens A and B The cups marked X contain both antigens for comparison

strains were being examined This difference can now be seen by the crossing (reaction of *non-identity*) of *bands C* and *D* Simultaneously, the merging (reaction of identity) of the band in front of cup Y with band C in front of the neighbouring cup proves that the antigen in the unknown strain *in cup Y is antigen C*

Figs 2 and *3* illustrate the *reactions* with two other sera Fig. 2 illustrates a case where the comparative strain in the cups marked X shows reactions with *two different antigens simultaneously* From Figs



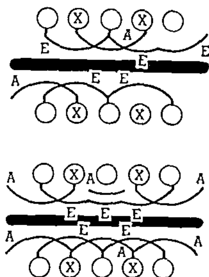


Fig 3
Same experiment as in Figs 1 and 2 with employment of still another donor serum containing antibody against antigen F Here the cups marked X contain antigen F for comparison

1 and 2 it may seem that the antigen A band covers other antigen bands It is evident from Fig 3, however, that the E band passes through the A band If the results are read directly instead of from photographs, other bands will be distinctly visible through the A band This, however, is difficult to reproduce photographically

The experiment shows that absorption can be entirely avoided by placing in every other cup a staphylococcus strain which is known to contain the antigen for which the unknown strains are to be examined

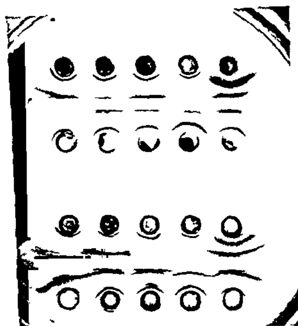


Fig 4

Gel precipitation reaction between serum from a patient with staphylococcal sepsis (in the long wells) and 20 different staphylococcal strains (in the cups) Reactions with at least seven different antigens can be seen

In this way reactions will occur that can be compared with the reactions given by the unknown strains in the other cups On the basis of reactions of identity and reactions of non identity with the known antigen bands from the comparative strains it can be determined whether the unknown strains contain the antigen in question With this technique a serum such as the one employed in the experiment illustrated in Fig 1 can be used in testing unknown strains for three different antigens since the reactions can be performed first with a comparative strain which contains antigen C then repeated with a comparative strain containing antigen D and finally with a comparative strain containing antigen A

Accordingly extracts were prepared of various strains each possessing a large amount of one of the eight antigens against which antibodies had been found These extracts were employed for comparative purposes As is evident from the illustrations four of the cups around each well will thus contain the comparative strains while the other six cups are left for the unknown strains

By means of this technique it was possible to examine the staphylococci for their content of eight different antigens These antigens have been designated as A B C D E F G and H With this method 161 strains of staphylococci have been examined Up to six of the eight

antigens were found to be present simultaneously in a few of the strains, whereas other strains contained only one or two antigens. Of the 161 strains cultivated from various patients with staphylococcal infections, 47 different combinations of the eight antigens were observed. It is not yet certain whether these 47 combinations represent 47 different types but preliminary investigations suggest that each strain presents the same antigenic structure from one subculture to another. There is, however, no doubt that a few of the antigens can vary in occurrence within the same staphylococcal strains, but such antigens may be ignored. On the other hand, previous investigations have shown that the staphylococci contain considerably more antigens than the 8 mentioned above, and that it is only a matter of examining a sufficiently large number of sera to find antibodies which demonstrate additional antigens. For this antigenic analysis it was considered appropriate to try to employ sera from patients with staphylococcal infections. An investigation shows that these patient sera contain antibodies more frequently and often in greater amounts than do sera from healthy persons. Sera from such patients, however, often simultaneously contain antibodies against numerous different staphylococcal antigens which makes them unsuitable for antigenic analysis. This is clearly seen from Fig. 4, which shows the reaction between serum from a patient with staphylococcal sepsis (in the long central well) and 20 different strains of staphylococci. Not less than seven different antigen-antibody bands are visible.

DISCUSSION

Gel-precipitation reactions with extracts of staphylococci have proved to be well-suited to the examination of human sera for staphylococcal antibodies. By this method the simultaneous occurrence of antibodies against as many as seven different staphylococcal antigens were demonstrated in sera from patients with staphylococcal infections (Fig. 4).

Similarly, staphylococcal antibodies have been demonstrated in sera from healthy donors. Apart from the normally occurring antibody against the staphylococcal antigen A (9) which is present in all human sera, demonstrable amounts of other staphylococcal antibodies were observed in 240 of 951 sera from healthy donors. Of these 240 sera seven could be used for examination of the antigenic structure of the staphylococci. With these seven sera it was practicable to demonstrate the occurrence of eight different staphylococcal antigens. A special technique is employed, whereby staphylococcal strains with a high content of a known antigen are used for comparison of the unknown strains, the nature of the antigens in the unknown strains being ascertained by means of reactions of identity and reactions of non-identity. In this way, absorption is entirely avoided. *Preliminary investigations*

show that the antigenic structure in the staphylococci is extremely complex. Of 161 staphylococcus strains examined 47 different antigenic combinations were observed with up to six different antigens occurring simultaneously in some strains. This fully explains why a satisfactory typing of the staphylococci is not practicable with other serological methods.

Whether it will be practicable to type the staphylococci by means of the method described in the present work is still uncertain. It can, however, be employed for elucidation of the problems associated with their typing, and it may also be used as a guide in connection with other serological methods.

Investigation of the antigenic structure of the staphylococci and the occurrence of staphylococcal antibodies in the serum of patients with staphylococcal infections may perhaps throw some light on the problems concerning the virulence of the staphylococci and the resistance of the patients.

A comparison between the antigenic structure of staphylococci and phage type, serological type by slide agglutination and antibiotic resistance pattern will likewise be of great interest. Investigation of these problems will be continued.

SUMMARY

As previously demonstrated, serum from healthy persons always contains a normally occurring antibody against the staphylococcal antigen A. Sometimes the serum from healthy persons also contains antibodies against other staphylococcal antigens. These antibodies can be demonstrated by means of the gel precipitation reactions with extracts of staphylococci.

By means of gel precipitation reactions and with employment of seven selected sera from healthy donors, an analysis has been made of [illegible] the staphylococci is exceed[illegible] [illegible] staphylococcus strains 47 different combinations of the eight antigens were observed. Up to six different antigens were found to occur simultaneously in some staphylococci.

In sera from patients with staphylococcal infections it was possible with the technique described in the present work to demonstrate antibodies against up to seven different staphylococcal antigens simultaneously. On account of their complex nature, sera from these patients do not lend themselves so well to an antigenic analysis of the staphylococci as do the selected sera from healthy persons.

REFERENCES

1 *Andersen, F Krag* Studies on the specificity of pemphigus staphylococci Acta path et microbiol scandinav *20* 242 1943

2 *Andersen F Krag & Heilesen B* On the occurrence and typing of staphylococcus pyogenes in weeping eczema (and the role played by these bacteria in the pathogenesis and course of eczema) Acta dermat-venereol *31* 679 1951

3 *Christie R & Keogh I A* Physiological and serological characteristics of staphylococci of human origin J Path & Bact *51* 189 1940

4 *Cohen J O Cowart, G S & Cherry W B* Studies of the antigenic relationship of staphylococci by the Ouchterlony technique Presented before the National Meeting of the Society of American Bacteriologists Philadelphia Pa May 2 1960

5 *Cowan S T* The classification of staphylococci by precipitation and biological reactions J Path & Bact *46* 31 1938

6 *Hegemann G* Untersuchung über Typendifferenzierung der Staphylokokken Zentralbl Bakt 1 Orig *140* 108 1937

7 *Hobbs B C* Studies of serological type differentiation of staphylococcus pyogenes J Hyg *46* 222 1948

8 *Jensen K* Investigations on the Antigenic Structure of Staphylococci Dissertation Munksgaard Copenhagen 1959

9 *Jensen, K* A normally occurring staphylococcus antibody in human serum Acta path et microbiol scandinav *44* 421 1958

10 *Julianelle L A* Studies of hemolytic staphylococci Hemolytic activity—biochemical reactions serologic reactions J Infect Dis *31* 256 1922

11 *Julianelle L A & Wieghard C W* Immunological specificity of carbohydrates derived from staphylococci Proc Soc Exper Biol & Med *31* 947, 1934

12 *Julianelle L A & Wieghard C W* Immunological specificity of staphylococci, occurrence of serological types J Exp Med *62* 11 1935

13 *Julianelle L A & Wieghard C W* Immunological specificity of staphylococci interrelationships of cell constituents J Exp Med *62* 31 1935

14 *Oeding P* Serological typing of staphylococci II Investigations on the serological chemical and physical properties of the antigens Acta path et microbiol scandinav *33* 312 1953

15 *Oeding P* Serological typing of staphylococci III Further investigations and comparison to phage typing Acta path et microbiol scandinav *33* 324 1953

16 *Oeding P* Immunochemical studies of staphylococcal polysaccharides 1 Methods for extraction and purification Acta path et microbiol scandinav *35* 484 1954

17 *Oeding P* Immunochemical studies of staphylococcal polysaccharides 2 The immunological properties of autoclaved extracts Acta path et microbiol scandinav *37* 116 1955

18 *Oeding P* Agglutinability of pyogenic staphyl cocci at various conditions Acta path et microbiol scandinav *41* 310 1957

19 *Pillet J Mercier P & Orta B* Recherches sur les agglutinines hétérologues des sérums antistaphylococciques I Utilisation d une méthode d absorption quantitative Ann Inst Pasteur *82* 465 1952

20 *Pillet J & Orta B* Recherches sur les agglutinines hétérologues des sérums antistaphylococciques II Étude de l agglutination par les sérums bruts Ann Inst Pasteur *82* 488 1952

21 *Pillet J & Orta B* Recherches sur les agglutinines hétérologues des sérums [illegible] de la spécificité des types 4 5 6 7 8 9

22 *Verw* [illegible] nic protein derived from the staphylococc [illegible]

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A NEW MEDIUM FOR THE BACTERIOLOGIC EXAMINATION OF STOOLS (LSU-AGAR)

By

Ingmar Juhlin and Carl Ericson

Received 30 xi 60

The amount of literature describing media for the isolation of entero pathogenic bacteria is immense (3, 6) Some of these media have become rather extensively used but none has offered undisputable advantages There is no such thing as a thorough understanding of the metabolism of the microorganisms concerned, nor of the pharmacological influences exerted on it by different substances, and this lack of knowledge reduces medium composition to a groping in the semi-darkness of empiricism While the supreme medium, built on a solid, scientific basis is still to be hoped for, there will be a continuous flow of new suggestions and modifications One will be described here

What demands should be put on an ideal medium for the bacterio logic examination of stools?

It must allow all the important entero-pathogenic bacteria (salmonellae shigellae, entero pathogenic coli, staphylococci) to grow freely while suppressing the growth of other microorganisms In practice this ideal has to be much modified However, the following claims on the medium would seem justified

(1) To suppress all growth of gram positive bacteria
(2) To suppress coli to a certain extent
(3) To suppress *Proteus* as much as possible and inhibit the swarming phenomenon completely
(4) To allow *Salmonella* species *Salmonella typhi* inclusive, and *Shigella* to grow freely
(5) [illegible]
(6) [illegible]
(7) [illegible]
(8) To be easily prepared without differing qualities in different batches

Bacteria other than *Salmonella* or *Shigella* but with the same inability to ferment the sugar ingredients of the medium should nevertheless be separable by means of the outward appearance presented by their colonies

Theoretical Motivations for the Choice of Test Substances

The choice of the tested inhibitors (sodium citrate, sodium desoxycholate, bile, sodium thiosulphate, sodium tetrathionate, potassium tetrathionate, crystal violet, magnesium chloride) is based on instructions obtained from manuals as to what substances are generally used for this purpose, and the approximate effect to be expected from them.

The choice of two sugars, lactose and saccharose, is promoted by the desire for a possible exclusion also of species that ferment saccharose but not lactose. In this way, the number of check-ups, such as fermentation tests in fluid media etc. may be lessened. The fact that some *Shigella* strains are able to ferment saccharose must then be specially considered.

The adding of urea is intended to simplify the separation of *Proteus* and other urease producers (1). It is true that urea is heat-labile, but as has been stressed by *Tidwell* (10) and *Suburth* (9), it is quite possible to effect a heat sterilization of a medium containing urea if proper regard is given to the fact that part of the urea is decomposed. With a suitable pH and buffering, this circumstance will not entail any disadvantages.

The system of indicators is constructed in such a way as to show distinct acid and alkaline changes (2). A system of two or more indicators is best capable of rendering a good result. The original colour of the medium is also of importance, and it is further desirable that the colonies themselves should take on clear and contrasting colours. Finally, the medium must be buffered in such a way that the indications, while being perfectly distinct, are limited to a comparatively small area near the pH changing colony.

EXPERIMENTAL

The Construction and Testing of Medium Varieties

Different combinations and amounts of ingredients have been scrutinized in a total of 54 medium varieties. For practical reasons a complete examination of every mathematically conceivable combination has not been made. The finally chosen variety was named LSU-agar (*Lactose, Saccharose, Urea*). In Tables 1 and 2 all the examined varieties are represented schematically. The test-strains used in the preliminary tests were *Salmonella typhi-murium*, *Salmonella enteritidis*, *Salmonella typhi*, *Shigella sonnei*, *Escherichia coli*, *Proteus sp*. Out of the combinations that were examined, the one described below proved to be the best in regard to the growth intensity of *Salmonella* and *Shigella*, the inhibition of coli, the inhibition of *Proteus* and its swarming, and distinct differences of colonial morphology and colour changes.

Certain observations may be of interest. Thus magnesium chloride proved too strong an inhibitor, and alcoholic crystal violet produced an inappropriate colour in the medium. Ox bile was difficult to handle and

TABLE 1
Combinations of Various Ingredients Tested

Ingredients	Amounts tested 1000 ml medium	Combinations 1	2	3	4	5	6	7	8	9	10	11	12	13	14	TSU
Di-sodium hydrogen phosphate × 2 H_2O	2.0 5.11 6.20 g	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
Lactose	15.0 20.0 40.0 g	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
Tri sodium citrate × 2 H_2O	2.0 g	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
Chrystal violet 1 per cent sol	0.5 ml	□	□	□	□	□	□	□	□	□	□	□	□			
Sodium desoxycholate	2.0 3.0 34.0 5.0 g			□		□	□	□	□	□	□	□	□	□	□	□
Urea	2.5 5.0 10.0 20.0 g							□		□	□	□	□	□	□	□
Potassium tetrathionate	0.5 1.0 7.5 10.0 g								□	□	□	□	□	□	□	□
Saccharose	20.0 40.0 g										□	□	□			□
Magnesium chloride × 6 H_2O	5.0 15.0 40.0 g		□			□						□	□	□		
Potassium-dihydrogen phosphate	4.34 5.18 g												□	□	□	□
Sodium tetrathionate	1.0 5.0 7.5 15.0 g	□	□	□												
Ox gall	75 ml	□	□		□											
Sodium thiosulphate × 5 H_2O	2.0 g				□	□	□	□								

TABLE 2
Indicator Systems Tested

Indicators	Amounts tested 1000 ml medium		Combinations 1	2	3	4	5	6	7	8	9	10	11	12	TSU
Methyl red	10 ml	1% sol in 50% alcohol	□	□	□		□	□	□						
Rosolic acid	10 ml	1% sol in 40% alcohol	□												
Cresol red	10 ml	1% sol in 96% alcohol		□	□										
Water blue	1 3 5 20 ml	2% sol in water			□	□		□	□	□	□	□	□	□	□
Phenol red	5 10 20 ml	1% sol in 60% alcohol				□				□		□			□
alfa Naphtolphtalein	10 ml	1% sol in 60% alcohol					□				□			□	□
Phenolphtalein	10 ml	1% sol in 50% alcohol						□					□		
Thymolphtalein	10 ml	1% sol in 50% alcohol							□			□			
para Nitrophenol	10 ml	1% sol in water								□					
Propyl red	2.5 5 10 ml	1% sol in 50% alcohol									□				

did not give better results than sodium desoxycholate Potassium tetrathionate is preferable to sodium tetrathionate owing to its better heat stability (3) A great amount of sugar enables weak fermenters to produce a comparatively swift change of colour The adjustment of the buffering effect proved essential for limiting the extension of the violet colour change especially Several combinations of indicators have been examined both in agar plates and in test tubes with buffers of different pH The finally accepted combination showed the most vivid and distinct colour changes The second best combination consisted of water blue, propyl red, and alpha-naphthol phthalein There was evidence however, that different batches of propyl red gave different results

LSU-agar

Ingredients

(1)	Bacto Beef Extract	5 0 g
	Proteose Peptone No 3 Difco	3 0 g
	Peptone UCLAF (Paris)	7 0 g
	Agar	21 0 g
	Aqua destillata	800 ml
(2)	Potassium tetrathionate Schuchardt München	0 5 g
	Urea Baker Phillipsburg N J	5 0 g
	Lactose	40 0 g
	Saccharose	40 0 g
	Aqua destillata	80 ml
(3)	Di sodium hydrogen phosphate × 2 H_2O Merck	6 20 g
	Potassium dihydrogen phosphate Merck	4 34 g
	Tri sodium citrate × 2 H_2O	2 0 g
	Aqua destillata	20 ml
(4)	Sodium desoxycholate Merck	2 0 g
	Aqua destillata	20 ml
(5)	Water blue 2 per cent solution Merck	3 0 ml
	Phenol red 1 per cent solution Merck	10 0 ml
	alpha Naphtolphtalein 1 per cent solution Merck	10 0 ml

Preparation

Ad (1) The nutrient agar is autoclaved at 122° C for 15 minutes after pH adjustment to 7 2

Ad (2) It is dissolved and desinfected in a water bath of 60° C for 1 hour

Ad (3) It is dissolved and desinfected in a water bath of 80° C for 1 hour

Ad (4) It is dissolved and desinfected in a water bath of 60° C for 1 hour

Ad (5) A 2 per cent solution of water blue 0 2 grams are dissolved in 10 ml of aq dest and are autoclaved at 122° C for 15 minutes

A 1 per cent solution of phenol red 1 0 gram is dissolved in 60 ml of 96 per cent alcohol and is heated in a water bath until seething after which 40 ml of boiling water are added

A 1 per cent solution of alpha naphthal phthalein 1 0 gram is dissolved in 60 ml of 96 per cent alcohol and this is heated in a water bath until seething after which 40 ml of boiling water are added The resulting medium has a pH of approximately 6 8 The colour is a light yellow brown It is transparent No further sterilization than that stated above is needed So far no primarily infected medium batches have been met with

Note The distilled water should be autoclaved at 122° C for 15 minutes in order to reduce its CO_2 content In this laboratory autoclaved distilled water has pH about 6 8

The Gross Morphology of Various Bacteria on LSU agar

After the preliminary examination with the above-mentioned test-strains, the said variety was chosen for further tests Various *Salmonella* and *Shigella* strains, 117 in all, were cultivated on the medium, together with a series of other enteric bacteria They are described in Table 3 The *Salmonella* and *Shigella* strains all grew copiously The colonial morphology etc of the different groups of enteric bacteria as observed in their growth on the medium may be studied in Table 4 The grouping of these bacteria is based on the results of sugar fermentations reported in Table 5

TABLE 3

Species of Salmonella and Shigella Studied on LSU Agar

Name	Number of strains
S paratyphi B	4
S typhi murium	71
S bredeney	1
S heidelberg	1
S montevideo	10
S oranienburg	5
S thompson	1
S bareilly	1
S muenchen	1
S manhattan	1
S bovis morbificans	1
S typhi	3
S enteritidis	2
S dublin	1
S panama	1
S muenster	1
S anatum	1
S amager	1
S cerro	1
S johannesburg	1
Sh flexneri	1
Sh sonnei	7
Total no of strains	117

The investigated *Salmonella* strains, except *Salmonella typhi*, all presented a uniform aspect separate colonies of 2–3 mm, that were clear, circular and had an entire edge, they were smooth, uncoloured, with or without a faint pink colour change in the medium (*Salmonella cerro* differed by possessing an irregular edge) After an incubation of 24 hours there was a distinct pink colour change surrounding a group of several colonies If streak inoculation is practised the growth is rich and confluent, translucent after 24 hours and presenting an entire edge, a finely granulated surface in its centre, and a moderate pink colour change (Plate 1) There is no trace of green, gray, blue, white, or violet, nor is there any precipitation of salts From the beginning, the whole

TABLE 4

Medium Changes and Morphology of Different Groups of Bacteria on ESU Agar

	Incub tim	Medium around colonies				Colony density			Colony colour			
		Precipitation	Pink zone	Violet zone	No change	Clear	Translucent	Opaque	Blue green yellow white	Pink	Violet	No colour
Salmonellae 26 strains	24 h		23		3	2	24		–	26	–	–
	48 h		26			–	26	–	–	26	–	–
Salmonella like 29 strains	24 h	3	13	1	12	11	16	2	4	20	–	5
	48 h	12	8	1	8	–	5	24	24	3	–	2
Non fermenters 20 strains	24 h		5	3	12	8	12	–	2	10	2	6
	48 h		9	5	6	3	8	9	11	5	1	3
Proteus morgani 12 strains	24 h			12	–	4	8		–	9	3	–
	48 h			12		1	11		–	–	12	–
Proteus vulgaris 4 strains	24 h	1		2	1	–	3	1	3	–	1	–
	48 h	2		1	1	–	1	3	–	–	1	–
Proteus mirabilis 6 strains	24 h	1		5	–	2	4		–	4	2	–
	48 h	1		5	–	–	5	1	2	–	4	–
Proteus rettgeri 1 strain	24 h			1	–	1	–	–	–	–	1	–
	48 h		–	1	–	–	1	–	–	–	1	–
Fermenters 22 strains	24 h	16	1	1	4	2	4	16	17	2	1	2
	48 h	21		1		–	1	21	22	–	–	–

TABLE 5

Fermentation Patterns etc of the Groups Referred to in Table 4
Numbers below Fermentation Signs Indicate Strains Giving that Reaction

	Arabinose	Xylose	Rhamnose	Dextrose	Galactose	Laevulose	Inositol	Maltose	Lactose	Saccharose	Raffinose	Inulin	Mannitol	Salicin	Indol	Hydrogen sulphide
Salmonella 37 strains	— 26	+ 34	+ 30	+ 37	+ 37	+ 37	— 27	+ 35	— 37	— 37	— 37	— 37	+ 37	— 34	— 37	+ 37
Salmonella like 39 strains	— 29	+ 35	+ 25	+ 39	+ 37	+ 37	— 39	+ 37	— 34	— 39	— 39	— 39	+ 39	— 29	+ 33	— 31
Non fermenters 43 strains	— 43	— 43	— 43	— 43	— 43	— 43	— 43	— 43	— 43	— 43	— 43	— 43	— 43	— 43	— 42	— 29
Proteus morgani 18 strains	— 18	— 18	— 18	+ 18	+ 17	+ 18	— 18	— 18	— 18	— 18	— 18	— 18	— 18	— 18	+ 17	— 18
Proteus vulgaris 6 strains	+ 3	+ 6	— 6	+ 6	+ 6	— 5	— 6	+ 4	— 6	+ 4	— 5	— 6	— 6	— 4	+ 6	+ 6
Proteus mirabilis 5 strains	— 3	+ 5	— 5	+ 5	+ 5	+ 4	— 4	— 5	— 5	— 5	— 5	— 5	— 4	— 4	— 5	+ 4
Proteus rettgeri 1 strain	— 1	— 1	+ 1	+ 1	— 1	+ 1	— 1	— 1	— 1	+ 1	— 1	— 1	+ 1	+ 1	+ 1	— 1
Fermenters 34 strains	— 24	+ 32	+ 24	+ 34	+ 33	+ 31	— 32	+ 33	+ 29	+ 22	— 19	— 31	+ 32	+ 17	+ 23	— 27

Fermentation medium nutrient broth with 1 per cent sugar
Brom thymol blue indicator   Incubated 5 days at 37° C

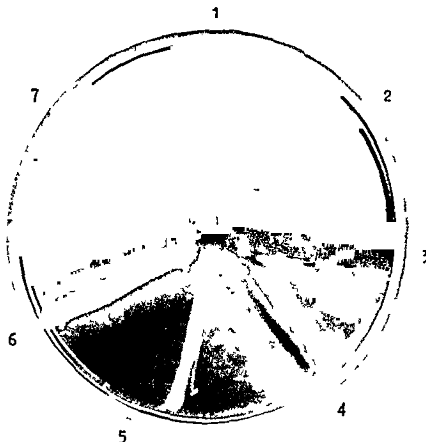


Plate 1

Streak cultures on ESU agar after 18 hours incubation at 37° C
1 Proteus mirabilis 2 Salmonella typhi murium 3 Pseudomonas
4 Escherichia coli 5 Shigella sonnei 6 Klebsiella 7 Salmonella typhi

culture gives an impression of pink After 2 3 days a faint yellow tinge may appear in the translucent centre, accompanied by a clear periphery and a pink colour change

In secondary streaks *Salmonella typhi* does not always show the pink colour change after 24 hours but it will appear after 48 hours (Plate 1) Its single colonies are often smaller than those of other *Salmonella* species, but otherwise their morphology is the same

After 24 hours, *Shigella* strains appear in separate colonies and in streaks as uncoloured and translucent, and they often present an irregular edge, a somewhat rough surface and no colour change After 48 hours both single colonies and streaks are opaque with a turquoise centre and a clear, irregular edge, showing no precipitates or colour changes (Plate 1) After three days the centre is opaque, lightening towards the uncoloured, translucent, outspreading edge A faint yellowish colour change is to be seen The fact that there is no precipitate constitutes a characteristic difference between *Shigella* and *Escherichia* or other fermenters

Those bacteria whose fermentation patterns show the closest similarities to that of *Salmonella* (*Arizona Bethesda Ballerup Alkalescens Dispar Providence* and others) may be indistinguishable from *Salmonella* after 24 hours in separate colonies In secondary streaks however there is a definite difference consisting of opaqueness a blue or green colour and a yellow or green precipitate These manifestations are probably caused by a weak and slow sugar fermentation that does not appear in the routine broth fermentation

Non fermentative bacteria (*Pseudomonas Alcaligenes*) in single colonies are sometimes impossible to distinguish from *Salmonella* at least if the incubation is only continued for 24 hours After this period the secondary streaks are generally pink or uncoloured without any colour change and with no granulation of the surface After 2 3 days the culture becomes opaque often yellow white greenish or grey sometimes markedly mucoid with a pink violet or no colour change There are no precipitates Even though the outward aspect does not differ much from that of *Salmonella Pseudomonas* is immediately recognizable by the tough and ropy consistency A quick and simple verification may be obtained by means of an oxidase test

With few exceptions lactose and/or saccharose fermenters even slow ones show a yellow or green precipitate in the medium after an incubation of usually only 24 hours Different species of bacteria present different types of colonies Escherichiae are a light or dark blue which may or may not turn paler towards the edge they are opaque and waxy Klebsiellae as a rule become mucoid yellow white and opaque while other species are different shades of grey yellow white and green

Turning to *Proteus* single saccharose fermenting strains here behave like the lactose and/or saccharose fermenters described above Owing to the effect of urease other *Proteus* strains produce a violet colour change in the medium Separate colonies in small quantities may after 24 hours present a *Salmonella* like appearance thus they are translucent and pink exhibiting an entire edge and a pink colour change The colonies are often small however No swarming occurs A prolonged incubation or a secondary streak is always accompanied by the strong violet colour change The culture is pink violet translucent smooth and has an entire edge

After 2 3 days incubation of secondary streaks of certain *Proteus* strains the violet colour next to the culture turnes out to disappear Sometimes there even is a yellow coloration and precipitates This phenomenon is more apparent after incubation at 20° C than at 37° C An investigation of 211 strains (Table 6) revealed this shift from a violet into a yellow colour change in the medium in 96 per cent of the *mirabilis* and 100 per cent of the *vulgaris* strains but not at all in *morgani* strains The explanation of this change toward greater acidity may be either a slow saccharose fermentation or a development of hydrogen

sulfide which are both to be seen in *mirabilis* and *vulgaris*, but not in *morgani*. Even though *Proteus* in small quantities will sometimes call for secondary streaks (see "Routine methods"), it is afterwards without exceptions easily distinguishable from *Salmonella*, and in no way complicates a diagnosis.

TABLE 6
Characteristics of Different Proteus Species on LSU Agar

Incubation		24 h 37°		48 h 37°		24 h 37°+24 h 22°		Total no. of strains
Medium colour		Violet	Yellow	Violet	Yellow	Violet	Yellow	
Prot. mirabilis	number	114	11	108	17	5	120	125
	per cent	91.2	8.8	86.4	13.6	4.0	96.0	
Prot. vulgaris	*number*	*3*	*4*	*2*	5	*0*	*7*	7
	per cent	42.9	57.1	28.6	71.4	0	100	
Prot. morgani	number	78	0	78	0	78	0	78
	per cent	100	0	100	0	100	0	
Prot. rettgeri	number	1	0	1	0	0	1	1

On the hypothesis that large amounts of *E. coli* on an LSU-agar plate would diminish the possibilities to recover small amounts of *Salmonella*, two tests were made. The results are given in Figures 1 and 2. In the first one (Fig. 1), suspensions of *E. coli* and *S. typhi-murium* were mixed in a tube series, where the ratio of *E. coli*-suspension to *S. typhi-murium*-suspension was varied after the schema, 10:0, 9:1, 8:2, 7:3, … 1:9, 0:10. From each tube 0.1 ml was spread on an LSU-agar plate with a 90 mm diameter. After 24 hours' incubation at 37° C, the colonies were counted. "Calculated numbers" are derived from the plate where only one kind of bacteria existed. In the figure, averages of two series are given. In the second test (Fig. 2), the method was similar, but the amount of *S. typhi-murium*-suspension in each tube was constant, and only the amount of *E. coli*-suspension varied. "Calculted amount" of *S. typhi-murium* in this test was obtained by the transfer of pure *S. typhi-murium*-suspension to LSU-agar. The results of both tests show that even very small amounts of *S. typhi-murium* can be recovered in expected numbers in the presence of large amounts of *E. coli*. Thus, all of the 16 calculated *S. typhi-murium* colonies were recovered in spite of the co-existence of more than 350 *E. coli* colonies on the plate. Although the test method used involves many pipettations and is consequently liable to give deviating results, the accordance between calculated and observed numbers is good in the pertinent parts of the curves (Fig. 1). This was the case also in the two individual series, on which the averages given in the figures are based.

calculated number of E coli
observed –"– " –"–
calculated –"– –" S typhi murium
observed –"– " –"–

Number of bacteria on the plates

0 1 2 3 4 5 6 7 8 9 10 ml S typh murium
10 9 8 7 6 5 4 3 2 1 0 ml E coli

Amounts of bacterial suspensions

Fig 1
Calculated and observed recovery rate on LSU agar inoculated from mixed suspensions with varying S typhi murium E coli ratios

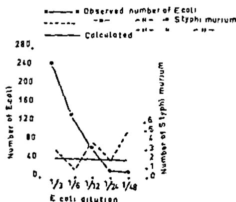


Fig 2
Recovery rate on LSU agar inoculated from mixed suspensions with varied amounts of E. coli but fixed S typhi murium concentration

A Comparison between LSU-Agar and other Media

In this laboratory, Endo agar was previously used for isolating *Salmonella* and *Shigella* from stools. For about 5 months, Endo agar and LSU-agar were in parallel use. The results of these experiments are given in Tables 7 and 8. It was found that the colonial morphology on LSU-agar enabled the observer to discard immediately far more samples than on Endo agar. The number of control tests with sugar fermentation etc. was thus considerably reduced (Table 7). With a growing experience of the medium, these controls can be almost entirely dispensed with.

The material includes 76 samples from 25 patients where at least one medium gave a positive result. In all these cases LSU-agar yielded positive cultures whereas 44 samples were negative on Endo agar (Table 8). This high frequency of negative samples on Endo agar may possibly have been technical rather than factual, since proteus overswarming in many cases made an estimation impossible.

TABLE 7

Comparison between Endo Agar and LSU Agar in Routine Use
Rate of Fermentation Controls in 684 Negative Specimens

	Specimens without suspect colonies				Fermentation controls			
	Endo agar		LSU agar		Endo agar		LSU agar	
	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
Primary plate	554	81.0	665	97.2	130	19.0	19	2.8
Secondary plate	536	78.4	601	87.9	148	21.6	83	12.1
Total	1090	79.7	1266	92.5	278	20.3	102	7.5

Secondary plate: inoculated from enrichment broths. Cf. Routine method in text.
Primary plate: direct inoculation from faeces.

TABLE 8

Comparison between Endo Agar and LSU Agar in Routine Use
Results in 76 Positive Specimens from 25 Patients

	Endo agar				LSU agar			
	Positive		Negative		Positive		Negative	
	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
Primary plate*	8	14.0	49	86.0	20	27.8	52	72.2
Secondary plate	32	42.1	44†	57.9	76	100.0	0	0

* Routine changed during one period no primary plating was done of specimens from known salmonellosis patients; during another primary Endo agar plates only were omitted in such cases. Primary and secondary plate: see note Table 7.

† Negative results in 28 cases connected with overswarming Proteus.

TABLE 9

Growth of Various Bacteria on some Selective Media

Organisms	Dilution	Blood agar		LSU agar		Desoxycholate citrate agar		SS agar	
		Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
Esch coli	1/100000	>500		>500		0		0	
	1/500000	278	100	152	54 7	0	0	0	0
Proteus	1/100000	>500		375		0		0	
	1/500000	325	100	130	40 0	0	0	0	0
S typhi	1/100000	>500		>400		130		0	
	1/500000	200	100	160	80 0	31	15 5	0	0
S typhi murium	1/100000	>500		>500		>500		101	
	1/500000	349	100	332	95 1	188	53 9	16	4 6
Sh sonnei	1/100000	>500		>400		14		0	
	1/500000	296	100	223	75 3	2	0 7	0	0

From seven (28 per cent) of the twenty-five patients, *Salmonella* was isolated on LSU-agar only Out of the remaining eighteen, two were diagnosed in earlier samples on LSU-agar than on Endo agar Even after the beginning of antibiotic therapy LSU-agar proved superior In 23 samples from 14 patients, which were taken while the treatment lasted or afterwards, *Salmonella* was isolated only on LSU-agar Consequently the risk that the patients leave hospital as carriers is considerably reduced if LSU-agar is used

Since Endo agar is not usually recommended nowadays as a medium for isolating *Salmonella*, a comparison was also made between LSU-agar and two media that are often prescribed in literature Desoxycholate citrate-agar (DC-agar) (made by the State Bacteriological Laboratory, Stockholm), and SS-agar (made by the Bacteriological Institute of Lund) The results can be seen in Table 9 which presents the average f

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sio ... made of broth cultures, 18 hours old, diluted 1/100,000 and 1/500 000 0 2 ml of each dilution of the different test bacteria were spread onto 90 mm plates with blood-agar, LSU-agar, DC-agar and SS-agar After an incubation of 24 hours at 37° C the number of bacteria was recorded As may be seen from the table, some 50 per cent of the coli were suppressed on the LSU-agar, but 100 per cent on the other two selective media The suppression of *Proteus* was somewhat stronger on the LSU-agar, being complete on the other two media here, too It emerged, however, that *Salmonella typhi*, too, was strongly inhibited by the other two selective media Thus no *Salmonella typhi* colonies were to be found on the SS agar, and only some 15 per cent on the

DC-agar, while the LSU-agar allowed 80 per cent of *Salmonella typhi* to grow as compared with the amount that could be isolated on the blood-agar In the case of *Salmonella typhi-murium*, too, LSU-agar is obviously the most advantageous, and this is even more true of *Shigella* The LSU-agar was consequently much less inhibitory than the SS or DC-agar to all the bacteria examined

This may seem to imply a certain disadvantage since undesirable bacteria are allowed to grow, but since *Proteus* swarming is totally suppressed, and the morphology of the various types of bacteria is very characteristic, this fact is of second importance only That coli should be allowed of a certain growth may even be of advantage, since enteropathogenic coli can consequently be isolated The essentially different possibilities of isolating *Salmonella*, especially *Salmonella typhi*, and *Shigella*, are so marked that there can be no doubt as to the superiority of the LSU-agar in this very important respect

Routine Methods for the Examination of Stools

For more than a year now, LSU-agar has been used in the routine work of this laboratory, and with good results The following methods have been employed The plates are dried for half an hour at 37° C It is of some importance that they should not get too dry A primary inoculation of stools is effected by means of parallel streaks—to secure single colonies—on LSU-agar and in 3 fluid media *Rappaport* (7, 8) and *Kauffmann* (3, 4) broths for the enrichment of salmonellae, and sodium chloride broth for the enrichment of staphylococci After 24 hours the two *Salmonella* enrichment broths are subcultured with parallel streaks to LSU-agar The sodium chloride broth is subcultured on blood-agar

The substitution of a more selective solid medium for the enriching broths is not recommendable (5) Not even when the solid medium is as tolerant as this one, is *Salmonella* always to be isolated on the primary plate It is a *sine qua non* for the isolation of *Shigella* that the primary plate should not be too strongly inhibitory To our experience, the enriching broths differ especially in that *Proteus* and *Pseudomonas* are much more effectively inhibited by the Rappaport broth On several occasions it has been possible to isolate *Salmonella* from one of the two enriching broths only There has been no particular examination as to which broth renders the best result but everything points to the advantages of a parallel use of the two broths

If there is a sufficient number of suspected colonies on the solid medium, an agglutination test is made directly If the number is less, secondary streaks of 5 10 colonies are made on LSU-agar After an incubation of 24 hours at 37° C it may easily be decided which cultures deserve a further investigation These cultures are subjected to agglutination tests, and a fermentation series may then sustain the diagnosis

As for the biochemical tests the most important items have turned out to be lactose mannitol saccharose the production of indole and hydrogen sulfide and finally the bacterial motility The advantages of a hydrogen sulfide test should be particularly stressed In those few cases where a 24 hour incubation of the secondary streaks does not permit a conclusive interpretation an incubation of another 24 hours nearly always clinches the matter definitely

A verbal tabular and photo pictorial description of what the various bacterial colonies look like on the medium does not give justice to the clear and easily distinguishable differences between *Salmonella Shigella* and other species An experienced diagnostician has no difficulty in analyzing the secondary streak cultures

DISCUSSION

The essential properties of the medium described above may be stated thus the advantages are the favourable growth conditions the palpably lessened need for broth fermentations demanding time and work the distinct and typical gross morphology of the cultures enabling a conclusive interpretation of secondary streaks so that a great number of suspected colonies can easily be examined The medium allows of a normal development of antigens A direct slide agglutination is made with perfect ease It is a striking point that even small amounts of *Salmonella* in a sample can be revealed by this *medium* This is of the utmost importance for the control of the therapeutic result If the medium is strongly inhibitory there is always the *risk* that the result of the treatment is regarded too optimistically *and in* consequence the patients leave hospital as carriers Furthermore patients under antibiotic treatment often have a large amount of *Proteus* in their stools and on certain media this makes the *isolation of Salmonella* extremely difficult or even *impossible* The *I SU* agar on the other hand inhibits any *Proteus* swarming completely and since the *Proteus* colonies moreover assume a violet colour *Salmonella* colonies can easily be distinguished

Like any other medium the I SU agar for its optimum use requires from the of some [illegible]

[illegible] ...what more and any swarming growth completely There are *distinct differences* in the colonial morphology of the various species of bacteria *Colour* changes and precipitates in the medium are clear and easily *interpreted* Combined with the secondary streak system these factors *provide* simplified and considerably enhanced possibilities to examine a *great number* of suspect colonies Urease producers as well as lactose and saccharose fermenters are easily distinguished from *Salmonella and Shigella* It is of special importance

that even a faint and slow sugar fermentation is noticeable in this medium LSU-agar is easy to prepare in such a manner that its properties remain constant throughout It may be kept in a refrigerator for at least two weeks, and still function satisfactorily

SUMMARY

A new medium (LSU-agar) is described, which is intended for the isolation of *Salmonella* and *Shigella* by inoculation from faeces directly and from enrichment broths The medium is buffered and contains large amounts of lactose and saccharose, urea, and a triple indicator system It is moderately inhibitory to *Escherichia coli* and *Proteus*, whose swarming is completely inhibited *Salmonella* species, including *S typhi* and *Shigella* grow freely without suppression of their antigen formation Colonial morphology and colour changes are distinctive In laboratory tests, the LSU-agar proved definitely superior to the Desoxycholate-citrate-agar and SS-agar in promoting the growth of *Salmonella typhi* and *Shigella sonnei* Parallel routine use of LSU-agar and Endo agar showed drastic differences Salmonella was demonstrated in 76 stool samples Positive results were obtained on LSU-agar in all the cases, while the isolation on Endo agar failed in 44 instances The superiority of LSU-agar was most evident after the onset of antibiotic therapy, when the amount of *Salmonella* diminished and *Proteus* increased

A suitable routine procedure for the isolation of *Salmonella* and *Shigella* in faeces is described in detail

REFERENCES

1 *Christensen W B* Urea decomposition as a means of differentiating Proteus and Paracolon cultures from each other and from Salmonella and Shigella types J Bact 52 461 1946
2 *Gintscheff P Z* Neue Differentialnahrboden fur die laufenden Untersuchungen Zbl Bakt I Abt Orig *173* 124 1958
3 *Hallmann L* Bakteriologische Nahrboden Georg Thieme Verlag Stuttgart 1953
4 *Kauffmann F* Weitere Erfahrungen mit dem kombinierten Anreicherungsverfahren fur Salmonella-Bacillen Zschr Hyg *117* 26 1936
5 *Legler F Burkhardt F & Ehrmann A* Beitrag zur Frage der optimalen Nahrbodenkombination in der Salmonella Diagnostik Zbl Bakt I Abt Orig *177* 559 1960
6 *Levine M & Schoenlein H W* A compilation of culture media for the cultivation of microorganisms Williams & Wilkins Baltimore 1930
7 *Rappaport F Konforti N & Navon B* A new enrichment medium for certain Salmonellae J Clin Path *9* 261 1956
8 *Rappaport F & Konforti N* Selective medium for paratyphoid bacteria Applied Microbiology 7 63 1959
9 *McNeill Sieburth J* Lactose urea agar a new differential tube medium for the detection of Salmonella J Bact *72* 278 1956
10 *Tidwell W L Heather C D & Merkle C* An autoclave sterilized medium for the detection of urease activity J Bact *69* 701 1955

FROM THE INSTITUTE OF CLINICAL BACTERIOLOGY (UNIVERSITY OF LUND)
GENERAL HOSPITAL MALMÖ HEAD PROFESSOR STEN WINBLAD M D

AN APPARATUS FOR THE RAPID AND UNIFORM STAINING OF BACTERIAL SMEARS

By

INGMAR JUHLIN

Received 16 xii 60

In the bacteriological laboratory a great number of slides with smears of clinical samples or cultures are stained every day Staining racks with a sandbox as overflow collector are still widely used Such and similar racks allow only individual and manual handling of each slide Much time can be saved if some kind of mechanization or collectivization is introduced, at least if the number of slides to be stained at the same time exceeds ten or fifteen Individual staining also leads to a varying quality as regards colour tones and contrasts, which is irritating to the microscopist and sometimes makes it impossible for him to make any judgment of the preparation It sometimes happens that slides fall down from the rack and get smashed against the floor or dirty in the sand The fingers and nails of the personnel are constantly discolored by manual handling of the overflowing slides

In order to eliminate these draw-backs and nuisances and at the same time considerably increase the capacity, a new staining rack has been devised (Fig 1 and 2) Supported by a robust metal construction (a), a bar (b) may be rotated by turning a knob (c) The knob is kept steady in four different positions by a spring loaded ball catch (e) mounted in a plate attached to the knob On the bar there are twelve slide holders consisting of metal springs (d) The size of the construction is adjusted to fit over the basins in the laboratory benches (Fig 2 b)

The slides are inserted in the holders and are kept horizontally while the stain is poured on All the slides are stained at the same time (Fig 2 b) Pouring off the stain is accomplished by turning the knob to a position at 60° angle In this position, the slides may also be rinsed with water If more stain is to be poured on, the slides are returned to the horizontal position otherwise they may be rotated to the vertical

[illegible] time in this

[illegible] This gives

[illegible] means safer judgment of the smears Manual treating of the slides can be avoided throughout the staining procedure,

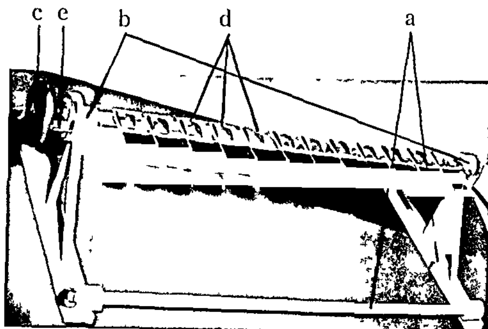


Fig 1
Staining rack a stand, b turnable bar, c knob, d metal springs



Fig 2a

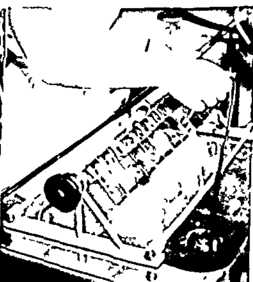


Fig 2b

The staining rack in use

which keeps fingers and nails of the technical assistants free from stain Finally, the staining capacity per unit of time is considerably increased, as all the slides on the rack can be treated simultaneously as a unit throughout the different stages of the staining procedure

FROM THE INSTITUTE OF CLINICAL BACTERIOLOGY (UNIVERSITY OF LUND) GENERAL HOSPITAL MALMÖ HEAD PROFESSOR STEN WINBLAD M D

SIMPLE SAFETY DEVICE FOR BUNSEN BURNERS IN BACTERIOLOGICAL LABORATORIES

By

INGMAR JUHLIN

Received 16 xii 60

In every bacteriological laboratory especially where training of technical assistants takes place there is always a risk of infections among the personnel caused by ignorance incompetence or accident Unskilled personnel is of course more exposed to such risks It is important to try out every possible device that may increase the safety in laboratory work

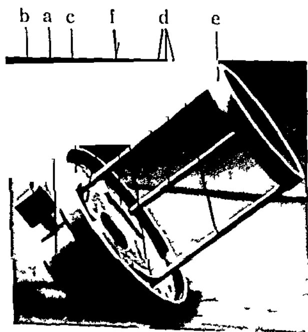


Fig 1

Safety device for Bunsen burners without glass cylinder a holder b screw c bottom plate d guiding strings e ring f curved elevations

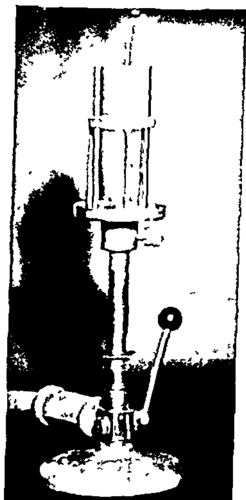


Fig 2
Bunsen burner with safety device in use

Sterilization of platinum wires in the Bunsen flame is done hundreds of times each day in bacteriological laboratory routine. With a correct technique this brings about a complete incineration of the infectious material. If the technical assistant is careless or untrained, however, an unsufficiently burned clot of infectious material may drop to the table or the floor or get caught on the clothes with potential possibilities of spreading and causing infections.

The following simple safety device has been constructed to avoid such accidents and has been used in this laboratory for some time (Fig 1 and 2). It consists of a holder (a) with a screw (b) for the attachment to the burner, and a circular bottom plate (c) welded to the holder and surrounded by a low wall. Three guiding strings (d) are welded to the bottom plate and are joined with a ring (e) at the top. A pyrex glass cylinder rests on three curved elevations (f) on the bottom plate.

The Bunsen burner tube is inserted through the hole in the holder (a) so that its upper end protrudes about a centimeter above the bottom

plate (c) The holder is fastened to the burner pipe with the screw (b) Air supply to the flame is secured through the openings between the elevations (f) on the bottom plate

The platinum wire is inserted into the flame inside the glass cylinder If any material is thrown away from the loop, it will stick to the hot glass or fall to the bottom plate where it is easily destroyed when desired

FROM THE INSTITUTE OF CLINICAL BACTERIOLOGY (UNIVERSITY OF LUND) GENERAL HOSPITAL MALMÖ HEAD PROFESSOR STEN WINBLAD MD

A MECHANIZED PROCEDURE FOR THE PREPARATION AND TUBING OF FLUID CULTURE MEDIA

By

INGMAR JUHLIN

Received 16 xii 60

Until cheap plastic tubes will become available, most laboratories still use glassware for the tubing of culture media Washing, drying, plugging with cotton-wool, tissue paper, or the like, and sterilization are extremely timeconsuming and thus expensive procedures In the preparation of media, especially such that do not stand autoclaving after tubing, there is the additional work of removing and reinserting plugs before and after the dispensing of the medium

To meet the much increased demand for tubed media of various kinds, a thoroughly revised preparation procedure has been introduced at this laboratory, with successful results To accomplish this, it has been necessary to furnish the laboratory with some new implements both commercially available ones and such of our own design

MATERIALS AND METHODS

1 Container made of perforated stainless steel The dimensions are 350 × 550 × 170 mm See Fig 1 It is divided into two compartments by a partition wall (a) parallell to its long axis Both long walls (b) are fastened with pegs (c) at the top *and with hinges (d) at the bottom so that they can easily be turned open* Inside the long walls there is in each compartment just above the bottom an adjustable L shaped bar (e) for the regulation of the inclination of the tubes On both sides of the partition wall there are vertical L shaped bars (f) which in combination with a system of *movable bars (g) hanging from metal cords (h) serve to separate* the tubes into sections and support them This arrangement allows the hot air or steam to penetrate freely into and among the tubes during sterilization (Fig 2)

2 Filamatic Vial Fillers Model AB 5 an automatical dispensing apparatus delivered by National Instrument Co Baltimore U S A

3 Metal tube caps CAP O TEST Ky 349 16/17 mm delivered by the firm Lüdi & Cie Flawil and Mogelsberg Switzerland They are available in five different colours (blue green red yellow silver)

Technique for Autoclavable Media

The container is packed with washed and dried test tubes, without previous plugging or wrapping Each compartment holds 232 (16/17 × 150 mm) tubes (8 layers of 29 tubes) The total capacity of the container is thus 464 tubes The arrangement of the tubes can be seen in

b d e g f h a c

Fig 1
Container of perforated stainless steel a partition wall b long wall c peg socket d hinges e L shaped bar f L shaped bar g movable bar h metal cord

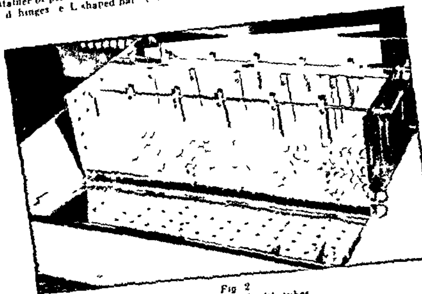


Fig 2
Container packed with tubes

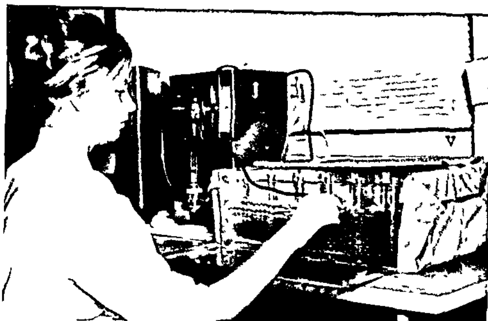


Fig 3
Container, packed with sterile tubes, during filling with sterile non-autoclavable medium

Fig 2 With the tubes in this position, the fluid medium is dispensed by a "Filamatic" Vial Filler In this way it is possible to supply every *tube in the container with 5 ml medium in about 7 minutes Thus, 3000* (Fig 3) portions of 5 ml may be tubed in less than an hour The metal tube caps, sterilized by dry heat or in the autoclave, are put on the tubes, which are transferred to string baskets and are autoclaved in the upright position in the customary way

Technique for Non-Autoclavable Media

The container is packed with washed dry empty tubes as before, but in 6 layers only, so that each compartment holds 174 tubes This is to ensure an optimal sterilization and to accomplish uniformity in the coagulation of Lowenstein-Jensen's, Loffler's, or similar media Thereafter, the container is wrapped in coarse paper and sterilized in dry heat at 175° C in 90 minutes After cooling, the wrapping paper is cut and folded out so that the long side can be opened and all tube mouths made visible Beforehand, the filling outfit plastic tubing, valve system syringe has been autoclaved Thus, the sterile, non-autoclavable medium can be transferred to the tubes through a closed sterile system The sterility of the tubes is maintained by means of their almost horizontal position, which minimizes the risk of infection with airborne bacteria (Fig 3)

If the medium is to remain fluid the sterile metal tube caps are put on and the tubes are then ready for use

If the medium is to be solidified by heat (Lowenstein Jensen Loffler or the like) the procedure is identical up to the point of filling the tubes with medium After this has been done the inclination angle of the tubes is adjusted by means of the L-shaped bar (Fig 1 e) The long walls are closed and locked with the pegs and the container is placed in an autoclave where coagulation of the medium is effected at 85°–89° C After that the tubes are plugged with sterile rubber stoppers or metal caps

By choosing different metal cap colours one can identify different tubed media with the same appearance omitting time consuming labelling Different media with identical use (*e g* enrichment broths for Salmonella) where the identity of each medium is obvious are preferably equipped with tube caps of the same colour

With the techniques suggested above the following advantages are gained

(1) Several re arrangements of the tubes on their way from the washing department *via* sterilization and medium dispensing to the autoclave are avoided

(2) The cumbersome plugging of the tubes with cotton wool can be omitted altogether

(3) There are no plugs to be removed before the dispensing of medium

(4) The medium is dispensed quickly with exact volume in every tube

(5) The dispensing of sterile media is much simplified and improved both through the inclination of the tubes and the existence of a protective "paper roof" and also by the use of metal caps fitted to the outside of the tubes instead of the inserting of cotton wool plugs

(6) The completely closed filling system further increases the safety when absolute sterility is essential

(7) At the coagulation of slants of Lowenstein Jensen s or Loffler s media the inclination of all the tubes in one compartment (174 tubes) can be simultaneously adjusted by simply turning a handle

(8) Time-consuming labelling of the tubes or their storage boxes can be avoided This ensures safe and easy identification as the mark of identity can not disappear by accident and can not remain longer than desired It will not happen that old pencil marks or paper labels confuse the identification

(9) Finally the suggested metal caps compare favourably with other commercially available types they are much easier to handle are at least as durable weigh a quarter and cost a third of some other brands



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AUS STATENS SERUMINSTITUT (INTERNATIONALE SALMONELLA- UND ESCHERICHIA-CENTRALE) KOPENHAGEN UND DER MEDIZINAL-UNTERSUCHUNGSANSTALT AM HYGIENISCHEN INSTITUT DER FREIEN UND HANSESTADT HAMBURG (LEITER: PROF. DR. MED. S. WINKLE)

NEUE BEFUNDE BEIM O-FORMEN-WECHSEL DER SALMONELLA-SPECIES

Von

F. KAUFFMANN und R. ROHDE

Eingegangen 5. XII. 60

Innerhalb der serologischen Variation spielt der „O-Formen-*Wechsel*" (O form-variation) eine wichtige Rolle, da ohne Kenntnis und Berücksichtigung dieser *dynamischen*, serologischen Vorgänge keine erfolgreiche Serologie betrieben werden kann. Der O-Formen-Wechsel oder kurz die „*O-Variation*" wurde zuerst von *F. Kauffmann* (1) beim O 1-*Antigen festgestellt*. Mit Hilfe der Objektglas-Agglutination kann man *bei zahlreichen species*, die das O 1-Antigen enthalten, Kolonien mit stark oder schwach entwickeltem O 1-Antigen, die einander abspalten, isolieren. In dergleichen Weise konnte *F. Kauffmann* (2) auch beim 12-*Antigen eine O-Variation* feststellen, und zwar beim 12_2-*Antigen*. Es treten Kolonien mit stark oder schwach entwickeltem 12_2-*Antigen auf* und spalten einander in wechselndem *Prozentsatze* ab. Enthalten *Salmonella*-Kulturen sowohl das O 1- als auch das 12_2-Antigen, so treten beide Formen-Wechsel *gleichzeitig und* unabhängig von einander auf:

$1++, 12_2++$
oder $1++, 12_2-\pm$
oder $1-\pm, 12_2++$
oder $1-\pm, 12_2-\pm$

Eine dritte Art der *O-Variation* wurde von *P. R. E*[illegible] ... Antigen, die einander abspalten nachweisen.

Obwohl damit gerechnet wurde, dass *auch bei* anderen *Salmonella*-O-Antigenen eine O-Variation zu *erwarten* sei, da verschiedene Unregelmässigkeiten und Schwierigkeiten *bei der Bestimmung* gewisser Antigen-Komponenten auftraten, so wurden bisher keine näheren Untersuchungen ausgeführt. Als aber derartige Störungen, speziell in den G- und H-Gruppen häufiger vorkamen, wurden Untersuchungen in dieser

At this laboratory, the methods and utensils described have been in use for almost two years During this time, the volume of media prepared has undergone a marked increase in spite of the fact that the number of people occupied with the preparation has remained unchanged As human beings in this country are far more expensive tools than the mechanical appliances described, the investment has doubtless proved to be sound

S grumpensis — 13 23 36 Es traten Formen mit stark oder schwach entwickeltem 36 Antigen die einander abspalteten auf War das 36 Antigen stark entwickelt so war die Objektglas Agglutination in 22 oder 23 Seren relativ schwach doch hangt dieses auch von der Starke der benutzten Seren ab Arbeitet man mit starker Faktor Seren so kann man diese Agglutinations Hemmung durchbrechen Zur Herstellung starker 36 Seren sind die 36++ Formen zu benutzen

1 37 Formen Wechsel zum Beispiel bei *S worthington* — 1 13 23 37 Dieser Formen Wechsel tritt gleichzeitig mit dem 1 Formen Wechsel auf sodass folgende Formen die einander abspalten vorkommen

1++ 37++ oder 1—± 37—±

Hat man daher den 1 Formen Wechsel bestimmt so hat man dadurch gleichzeitig auch den 37 Formen Wechsel erfasst Zur Herstellung starker 37 Seren sind die 37++ Formen zu wahlen

Bei *species* mit 1 36 und 37 Antigenen zum Beispiel bei *S mississippi* = 1 13 23 36 37 kommen alle 3 Formen Wechsel 1 36 und 37 vor wobei der 36 Formen Wechsel unabhangig vom 1 37 Formen Wechsel verlauft Es konnen folgende Formen die einander abspalten auftreten

1++ 36++ 37++
oder 1++ 36—± 37++
oder 1—± 36++ 37—±
oder 1—± 36—± 37—±

Unter Berucksichtigung dieser Formen Wechsel wurden die Antigen Formeln folgender *species* kontrolliert und konnen wie folgt angegeben werden

S poona = 13 22 36
S willemstad = 1 13 22 37
S rotterdam = 1 13 22 36 37
S tanger = 1 13 22 36 37
S grumpensis = 13 23,36
S atlanta = 13 23 36
S worthington = 1 13 23 37
S mississippi = 1 13 23 36 37

Wahrend wir mit diesen Untersuchungen uber den O Formen Wechsel beschaftigt waren teilte Dr *B Stocker* London brieflich mit dass er unabhangig von uns einen kombinierten 1 37 Formen Wechsel beobachtet habe

6 14 24 und 1 25 Formen Wechsel

In der *Salmonella* II Gruppe wurden folgende Formen Wechsel gefunden

Richtung vorgenommen und sollen im folgenden kurz beschrieben werden

1–27–Formen-Wechsel.

In der *Salmonella* B-Gruppe konnte ausser dem oben erwähnten 1- und 12_2-Formen-Wechsel noch ein *27-Formen-Wechsel* nachgewiesen werden, z B bei *S bredeney* und anderen *species* mit der Formel 1,4, 12,27 Es traten folgende Formen, die einander abspalteten, auf

1 + +, 27—± oder 1—±, 27 + +.

Das Zeichen + + bedeutet in allen, diesen Formeln, dass das betreffende Antigen sehr stark entwickelt ist, sodass es schnell zu einer sehr starken Objektglas-Agglutination, die mit blossem Auge zu erkennen ist, kommt Das Zeichen —± bedeutet, dass nur langsam eine schwache Objektglas-Agglutination, die mit der Lupe sichtbar ist, auftritt Auch in der Reagenzglas-Agglutination mit abgetöteten Kulturen lassen sich deutliche, quantitative Unterschiede zwischen diesen beiden Formen feststellen, sowohl betreffs Titer als auch Stärke der Agglutination

Wie es aus obigen Formeln 1 + +, 27—± oder 1—±, 27 + + hervorgeht, kann man das 27-Antigen nur dann gut nachweisen, wenn man eine 1—± Form benutzt Ebenso muss man zur Erzielung eines starken O 27-Serums zur Immunisierung eine 1—±, 27 + + Form anwenden

Bei *S schleissheim* = 4,12,27 konnte bisher kein 27-Formen-Wechsel festgestellt werden, alle untersuchten Kolonien lagen in der 27 + + Form vor Daher eignet sich diese *species* besser zur Herstellung eines 27-Serums als die 1,4,12,27-Kulturen, bei denen ein Formen-Wechsel auftritt

1 19 Formen-Wechsel

Während also bei *S bredeney* und anderen *species* die 1- und 27-Antigene alternierend stark oder schwach entwickelt auftraten, d h in 1 + +, 27—± oder 1 ±, 27 + + Formen, so konnte in der E-Gruppe bei *S chittagong* = (1),3,10,(19) folgender kombinierter 1-19 Formen-Wechsel nachgewiesen werden

1 + +, 19 + + oder 1 —±, 19—±

Et traten hier also Kolonien, bei denen beide Antigene gleichzeitig stark oder schwach entwickelt waren auf Bei den 3- und 10-Antigenen konnte kein Formen-Wechsel gefunden werden

1 36 37-Formen-Wechsel

In der *Salmonella* G-Gruppe wurden folgende O-Variationen nachgewiesen

36-Formen-Wechsel, zum Beispiel bei *S poona* = 13,22,36 und bei

1++, 25++ oder 1—±, 25—±

Hat man daher den 1 Formen-Wechsel bestimmt, so hat man damit gleichzeitig den 25 Formen-Wechsel erfasst Dieses ist bei der praktischen Bestimmung des 25-Antigens in der Objektglas-Agglutination von Bedeutung, da man zum Nachweis des 25-Antigens eine 1++ Kolonie wahlen muss Ebenso ist es nötig, zur Herstellung eines 25-Serums eine 25++ Kultur zu benutzen Lagen die 1++, 25++ Formen vor, so war das 6-Antigen oft schwächer nachweisbar, im Gegensatz zu den 1—±, 25—± Formen, doch konnte kein typischer 6-14-Formen Wechsel festgestellt werden Man muss jedoch damit rechnen, dass diese Resultate bei verschiedenen *species* oder verschiedenen Kulturen derselben *species* varıieren konnen, sodass sich keine allgemeingültigen Regeln aufstellen lassen

BESPRECHUNG DER ERGEBNISSE

Die oben geschilderten Resultate, die weitere O *Variationen* (oder O Formen-Wechsel) bei verschiedenen *Salmonella species* ergaben, machen es wahrscheinlich, dass wir es bei der *O-Variation* mit einer allgemeinen, weit verbreiteten Erscheinung in der Serologie der *Entero bacteriaceae* zu tuen haben Speziell innerhalb des *genus Salmonella* kommen so zahlreiche *O-Variationen* vor, dass wir nicht nur bei den bisher untersuchten Antigenen mit einer derartigen Variation zu rechnen haben, sondern auch bei anderen Antigenen

Da diese *O-Variationen* besonders häufig innerhalb der *Salmonella* A- B- und D-Gruppe auftreten (1- und 12 Formen-Wechsel), so wird es verständlich, dass eine Standardisierung der Widal-Reaktion ohne Berucksichtigung dieser Formen-Wechsel zu Misserfolgen führen muss Die Starke und der Titer bestimmter Reaktionen hängen nämlich davon ab ob bestimmte Antigene stärker oder schwacher entwickelt sind Ferner werden Antigene, die nicht selbst einem O Formen-Wechsel unterworfen sind durch die stärkere oder schwächere Entwicklung bestimmter Antigene beeinflusst Besonders deutlich wurde dieses in der *Salmonella* G-Gruppe bei der Bestimmung der 22- und 23 Antigene

Um aber alle hierbei auftretenden Probleme zu lösen, wären weitere, eingehende Untersuchungen erforderlich Man muss sich darüber klar sein dass der O Formen-Wechsel bei allen serologischen Bestimmungen der Korper-Antigene eine grosse Rolle spielen kann, und dass mit dem Vorkommen weiterer, bisher unbekannter Formen-Wechsel zu rechnen ist

Über den zu Grunde liegenden Mechanismus des O-Formen-Wechsels ist bis heute nichts bekannt, doch kann soviel gesagt werden, dass es sich nicht um Verlust-Varianten oder um S-R-Formen-Wechsel handelt, sondern um eine besondere Form der Variation Auffallend ist der hohe Prozentsatz der Abspaltungen, der in der Regel zwischen 5 20 %

6-14-24-Formen-Wechsel, z B bei *S carrau* Es traten folgende Kolonien, die einander abspalteten, auf:

6+ +, 14+ +, 24+ +
und 6—±, 14—±, 24—±

Ein derartiger Formen-Wechsel, der alle 3 Partial-Antigene gleichzeitig betraf, wurde erstmalig festgestellt Aus diesem Grunde wurden auch die 3 übrigen *species* mit 6,14,24-Antigenen näher untersucht, und zwar *S albuquerque*, *S heves* und *S lindern*

Bei *S albuquerque* wurde das O 1-Antigen mit 1-Formen-Wechsel gefunden, unabhängig vom 6 14-24-Formen-Wechsel Die O-Formel von *S albuquerque* ist daher auf 1,6,14,24 zu erweitern

Bei den beiden anderen *species* *S heves* und *S lindern* konnte das früher eindeutig nachgewiesene 24 Antigen jetzt nicht mehr gefunden werden, sodass dieses Antigen also verloren gegangen ist Dieser Befund bildet eine Parallele zu *S boecker* = 6,14 : l,v 1,7, bei der von Anfang an kein 24-Antigen gefunden werden konnte Es muss aber damit gerechnet werden, dass Kulturen von *S boecker* mit der Formel 6,14,24 l,v : 1,7 angetroffen werden, sodass in diesem Falle keine neue *species* zu errichten wäre

14 24-Formen-Wechsel

Bei *S boecker* konnte ein 6-14-Formen-Wechsel festgestellt werden, da Kolonien mit 6+ +, 14+ + oder 6 ±, 14 ±, die einander abspalteten, auftraten Gelegentlich kamen auch Kolonien der Form 6—±, 14+ + vor

Von den beiden Formen 6+ +, 14+ + und 6 ±, 14 ± wurden Kaninchen-Seren hergestellt und in Absorptionsversuchen geprüft Beide Seren konnten durch *S carrau* = 6,14,24 völlig erschöpft werden, sodass also *S boecker* keinen Sonderfaktor enthält Durch Absorption mit *S thompson* konnten reine 14-Seren hergestellt werden Diese Faktor-Seren ergaben in der Objektglas-Agglutination mit allen Kulturen, die das O 14-Antigen besassen, eine prompte und starke Objektglas-Agglutination

Da also bei 6,14,24-Kulturen abgesehen vom 6 14 24-Formen-Wechsel, auch Verlust-Varianten ohne das 24-Antigen vorkommen, so sollte man auf den Nachweis des 24-Antigens kein all zu grosses Gewicht legen und sich bei der O-Antigen Bestimmung derartiger Kulturen eventuell mit dem Nachweis der 6 und 14-Antigene begnügen

1 25 Formen-Wechsel

Dieser Formen-Wechsel wurde bei *S onderstepoort* und anderen *species* mit den O-Antigenen (1),6,14,25 festgestellt Es traten gleichzeitig mit dem 1-Formen-Wechsel folgende Formen, die einander abspalteten, auf

COMMUNICABLE DISEASE CENTER PUBLIC HEALTH SERVICE U S DEPARTMENT OF HEALTH EDUCATION AND WELFARE ATLANTA GEORGIA AND STATENS SERUMINSTITUT (INTERNATIONAL SALMONELLA AND ESCHERICHIA CENTER) COPENHAGEN

FOUR NEW SALMONELLA SPECIES· S. ALAMO, S NEW-MEXICO, S. WAYNE AND S. MARICOPA

By

P R EDWARDS, F KAUFFMANN and MARY A FIFE

Received 15 xii 60

The four *species* to be described were received at the Communicable Disease Center over a period of years and because of their biochemical and serological properties were catalogued as intermediate cultures related to *Salmonella* and *Arizona*

However, when the work of *Schaub* on malonate utilization by *Arizona* cultures, the work of *Kauffmann & Petersen* on the action of *Salmonella* and *Arizona* cultures on D-tartrate, citrate and mucate, and the confirmation of both of these observations by *Ellis, Edwards & Fife* were considered, it became obvious that the cultures definitely must be placed in the *genus Salmonella*

The cultures in question all possessed biochemical characteristics typical of *Enterobacteriaceae* In addition they were motile, with one exception failed to produce indol, were methyl red positive and Voges Proskauer negative, and grew readily on ammonium citrate agar Nitrate was reduced, urease was not produced, hydrogen sulfide was formed gelatin was liquefied, and lysine and ornithine decarboxylase and arginine dihydrolase were produced D tartrate, citrate, and mucate media were promptly acidified but malonate was not utilized and no growth occurred in KCN medium L-tartrate and i tartrate were not fermented Glucose and mannitol were fermented promptly with gas production Acid was formed from arabinose, xylose, rhamnose, maltose, dulcitol and sorbitol within 24 hours Lactose, sucrose, salicin and adonitol were not fermented One of six cultures of *S new mexico* fermented inositol This was the *culture which produced indol* The one available culture of *S alamo* also fermented inositol *S wayne* and *S maricopa* did not ferment inositol Failure to ferment lactose, prompt fermentation of dulcitol and utilization of D-tartrate, citrate and mucate, and failure to utilize malonate indicated that the organisms were salmonellae and not members of the *Arizona* group

In describing the serological properties of the four *species*, it seems best to discuss the H antigens of the cultures jointly since *S alamo*,

oder höher liegt. Dieser Prozentsatz entspricht ungefähr demjenigen beim *Phasen-Wechsel* innerhalb der H-Antigene, sodass auch beim *O-Formen-Wechsel* eine genetisch bedingte Reaktion vorliegen muss.

ZUSAMMENFASSUNG

Nachdem bereits früher ein *O-Formen-Wechsel* bei den Antigenen 1, 6_1 und 12_2 festgestellt war, wurden weitere *O-Variationen* (= O-Formen-Wechsel) bei folgenden Körper-Antigenen des *genus Salmonella* nachgewiesen.

1. *1–19-Formen-Wechsel* bei *S. chittagong* =
   1 + +, 19 + + oder 1—±, 19—±
2. *6–14–24-Formen-Wechsel* bei *S. carrau* =
   6 + +, 14 + +, 24 + + oder
   6—±, 14—±, 24—±
3. *1–6–14–24-Formen-Wechsel* bei *S. albuquerque*,
   unabhängig vom 6–14–24 Formen-Wechsel kam es zu einem 1-Formen-Wechsel: 1 + + oder 1—±
4. *6–14-Formen-Wechsel* bei *S. boecker* =
   6 + +, 14 + + oder 6—±, 14—±
5. *1–25-Formen-Wechsel* bei *S. onderstepoort* =
   1 + +, 25 + + oder 1—±, 25—±
6. *1–27-Formen-Wechsel* bei *S. bredeney* =
   1 + +, 27—± oder 1—±, 27 + +
7. *36-Formen-Wechsel*, z. B. bei *S. poona* und *S. grumpensis* =
   36 + + oder 36—±
8. *1–37-Formen-Wechsel*, z. B. bei *S. worthington* =
   1 + +, 37 + + oder 1—±, 37—±

Unabhängig hiervon kam es bei *S. mississippi* = 1,13,23,36,37 gleichzeitig zum 36-Formen-Wechsel, sodass also ein *1–36–37-Formen-Wechsel* vorliegt.

Es wird auf die Bedeutung dieser O-Variationen für die Serologie der *Salmonella*-Gruppe eingegangen und darauf hingewiesen, dass wir von einer vollständigen Klarstellung der serologischen Variation noch weit entfernt sind.

LITERATUR

Edwards, P. R.: Form variation in group C Salmonella strains. Proc. Soc. Exper. Biol. and Med. *59*, 49, 1945.

Kauffmann, F. (1): Zur Serologie des 1-Antigens in der *Salmonella*-Gruppe. Acta path. et microbiol. scandinav. *17*, 135, 1940.

Kauffmann, F. (2): A typhoid variant and a new serological variation in the *Salmonella* group. J. of Bacter. *41*, 127, 1941.

Stocker, B.: Persönliche Mitteilung, 1960.

would require rearrangement of established absorption procedures for the production of single factor p serum so that the antigens in question would not react with it

A second and simpler solution is to assign the symbols g, (p) to the antigens to indicate that the organisms contained a portion of antigen p of *S dublin* and it was this method of designation which was adopted The phase 2 antigens of *S alamo*, *S new mexico* and *S maricopa* were closely related to the 1,5 phases of the *Salmonella genus* and reacted to the titres of various 1,5 sera and of single factor 5 serum They did not effect a complete removal of agglutinins from sera prepared from phase 2 of *S thompson*, *S cholerae-suis* or *S decatur*, all of which possess 1,5 phases Further, the phase 2 antigens of *S alamo*, *S new mexico* and *S maricopa* each contained small specific fractions not present in the others It is well known that slight differences exist in many of the 1,5 phases of the *Salmonella genus* and phase 2 of the organisms was designated simply as 1,5

S alamo was represented by one culture received from Lt Col P R Carlquist and was isolated from the stool of an adult affected with diarrhea It was agglutinated to the titre of S thompson (6,7) O serum and in absorption tests removed all agglutinins from the serum The antigenic formula was 6,7 g,(p) 1,5

S new mexico was represented by five cultures, two recovered from the stools of patients affected with gastro-enteritis and three recovered from sewage All were agglutinated to the titre of *S gallinarum* (9,12) serum and in absorption tests removed all agglutinins from the serum The antigenic formula was 9,12 g,(p) 1,5

S wayne was represented by one culture recovered from the intestine of a lizard in the Detroit Zoo The organism was agglutinated to the titre of *S urbana* (30) O serum In absorption tests the titre of the serum was reduced 50 per cent Likewise, serum prepared from *S wayne* agglutinated *S urbana* to titre but absorption of the serum by *S urbana* resulted only in a loss of 50 per cent of the titre The O antigens of *S wayne* were designated simply as 30 and the antigenic formula was 30 g (p)

S maricopa was represented by one culture isolated from sewage The culture possessed *Salmonella* O antigen 1 and was agglutinated to the titre of *S weslaco* (42) O serum [illegible]
were removed from [illegible]
serum by *S weslaco* [illegible]
from the serum The [illegible] formula was 1,42 g,(p) 1,5

Dr *P R Edwards* is regarding the four published *Salmonella species* as sero types

S new-mexico, and *S maricopa* were diphasic and phases 1 and 2 respectively were closely related in all while *S wayne* was monophasic and phase 1 was closely related to phase 1 of the other types The phase 1 antigens were flocculated in very low dilution by *Salmonella* sera that contained agglutinins for antigen g , i e *S enteritidis*, *S dublin* *S derby* and related forms Agglutination also occurred in low dilutions of single factor p serum but not in sera for factors f, m, q, s, or t Flocculation occurred to the titres of *Arizona* H 13,14 and H 13,15 sera and in single factor serum for H 14 When sera were prepared from phase 1 of the four types, *S dublin* was flocculated to approximately half the titres of the sera and other salmonellae containing related antigens to lower titres The reactions obtained with phase 1 serum of *S alamo* are illustrated in Table 1 Similar results were obtained with sera of the other *species* Reciprocal absorption tests revealed that, while the phase 1 antigens of the four *species* were closely related, none was identical After absorption a residue of agglutinins amounting to approximately 1 per cent of the original titres remained in the sera

TABLE 1

H Antigens of S alamo Phase1

Sera	Antigens							
	S alamo phase 1	*S new mexico* phase 1	*S wayne*	*S maricopa* phase 1	*Arizona* H13 14 (NJ 4)	*S dublin* (g p)	*S derby* (f g)	*S enteritidis* (g m)
S alamo phase 1								
Unabsorbed	12800	6400	12800	12800	12800	6400	400	1600
Absorbed by								
S wayne	*200*	*<100*	*<100*	*<100*	*<100*	*<100*	*<100*	*<100*
Arizona H 13 14	1600	200	400	200	<100	<100	<100	<100
S dublin	3200	1600	3200	1600	1600	<100	<100	<100
Arizona H 13 15								
Unabsorbed	12800	12800	12800	12800	25600	6400	800	1600
Absorbed by								
S alamo	<100	<100	<100	<100	1600	<100	<100	<100
S dublin	1600	800	800	1600	3200	<100	<100	<100
S dublin								
Unabsorbed	100	400	400	400	400	25600	800	3200
Absorbed by								
S alamo	<100	<100	<100	<100	<100	6400		

— = Tests not done

Two solutions to the problem of assigning symbols to phase 1 of these four *species* are possible They could be assigned the symbol g together with a second symbol to denote the antigens present which previously have not been catalogued in the *Kauffmann-White-schema*, and which would be more or less synonymous with *Arizona* H antigen 14 This

would require rearrangement of established absorption procedures for the production of single factor p serum so that the antigens in question would not react with it

A second and simpler solution is to assign the symbols g (p) to the antigens to indicate that the organisms contained a portion of antigen p of *S dublin* and it was this method of designation which was adopted The phase 2 antigens of *S alamo, S new mexico* and *S maricopa* were closely related to the 1,5 phases of the *Salmonella genus* and reacted to the titres of various 1,5 sera and of single factor 5 serum They did not effect a complete removal of agglutinins from sera prepared from phase 2 of *S thompson, S cholerae-suis* or *S decatur,* all of which possess 1,5 phases Further, the phase 2 antigens of *S alamo, S new mexico* and *S maricopa* each contained small specific fractions not present in the others It is well known that slight differences exist in many of the 1,5 phases of the *Salmonella genus* and phase 2 of the organisms was designated simply as 1,5

S alamo was represented by one culture received from Lt Col P R Carlquist and was isolated from the stool of an adult affected with diarrhea It was agglutinated to the titre of *S thompson* (6,7) O serum and in absorption tests removed all agglutinins from the serum The antigenic formula was 6 7 g,(p) 1,5

S new mexico was represented by five cultures, two recovered from the stools of patients affected with gastro enteritis and three recovered from sewage All were agglutinated to the titre of *S gallinarum* (9,12) serum and in absorption tests removed all agglutinins from the serum The antigenic formula was 9,12 g,(p) 1,5

S wayne was represented by one culture recovered from the intestine of a lizard in the Detroit Zoo The organism was agglutinated to the titre of *S urbana* (30) O serum In absorption tests the titre of the [illegible]

[illegible] designated simply as 30 and the antigenic formula was 30 g (p)

S maricopa was represented by one culture isolated from sewage The culture possessed *Salmonella* O antigen 1 and was agglutinated to the titre of *S weslaco* (42) O serum In absorption tests all agglutinins were removed from the serum Likewise, absorption of *S maricopa* O serum by *S weslaco* and *S paratyphi A* completely removed agglutinins from the serum The antigenic formula was 1,42 g,(p) 1,5

Dr *P R Edwards* is regarding the four published *Salmonella species* as sero types

SUMMARY

Four new *Salmonella species* are described All possess similar antigens in phase 1 and attention is called to the close relationships of these antigens to the *Arizona* H antigens 13,14 The organisms and their antigenic formulas are as follows

S alamo = 6,7 g,(p) 1,5, *S new-mexico* = 9,12 g,(p) 1,5
S wayne = 30 g,(p) — and *S maricopa* = 1,42 g,(p) 1,5

REFERENCES

Ellis R J Edwards P R & Fife M A The differentiation of the *Salmonella* and *Arizona* groups by utilization of organic acids Pub Health Lab *15* 89 1957
Kauffmann F & Petersen A The biochemical group and type differentiation of *Enterobacteriaceae* by organic acids Acta path et microbiol scandinav *38* 481 1956
Schaub I G Cultural differentiation of paracolon bacilli Bull Johns Hopkins Hosp *83* 367 1948

FROM STATENS SERUMINSTITUT (INTERNATIONAL SALMONELLA AND ESCHERICHIA CENTER) COPENHAGEN

SUPPLEMENT TO THE KAUFFMANN-WHITE-SCHEME (IV)

By

F. KAUFFMANN

Received 29.xii.60

This paper is the 4. supplement to the review '*Das Kauffmann White-Schema*' (1) containing 62 *Salmonella species* recognized during 1960. From these 62 *species* 47 belong to *sub genus* I and 15 to *sub genus* II (*Kauffmann* 2). The *species* of *sub genus* II are indicated in the following table by an asterisk (*). Deviating biochemical results, not given in the table, were obtained in the following *species*.

S. flottbek, *S. langenhorn* and *S. othmarschen* were KCN positive. Further *S. flottbek* was sorbitol negative.

S. kumasi, *S. lawndale* and one culture of *S. new mexico* were indol positive.

S. ferlac was sorbitol negative and lactose positive.

Salmonella 16: z_4, 1 (5) 7 was salicin positive.

The other biochemical results are given in the table.

According to the definition "*A species is a group of related sero fermentative phage types*" (*Kauffmann* 3) the following types, either they are published as "*species*" or not, are regarded as *species* of the *genus Salmonella*.

Please turn over

Species	Ara	Dul	Ino	Rha	Tre	Xyl	Gly	H_2S	Gel	d	l	i	Cit	Muc	Mal
Salmonella = 4 12 b 1 6	+	+	—	+	+	+	++	+	—	+	×	—	+	+	—
•S bechuana = 4 12 27 g t	+	+	—	+	+	+	++	+	+	—	—	—	+2	+	+
S ruki = 4 5,12 y e n x	+	+	—	+	+	+	++	+	—	+	+	×	+	+	—
S albert = 4 12 z_{10} e n x	+	+	—	+	+	+	++	+	—	+	×	—	+	+	—
S wilhelmsburg = 4 12 27 z_{38}	+	+2		+	+	+	++	+	—	—	—	—	+	+	—
S coleypark = 6 7 a 1 w	+	+	—	+	+	+	++	+	—	+	×	—	+	+	—
S othmarschen = 6 7 g m t	+	+	+	+	+	+	++	+	—	+	×	—	+	+	—
S alamo = 6 7 g (p) 1 5	+	+	+	+	+	+	++	+	+	—	—	—	+	+	—
S gdansk = 6 7 l v z_6	+	+	—	+	+	+	++	+	—	+	+	—	+	+	—
•Salmonella = 6 7 z_{39} 1 7	+	+	—	+	+	+	++	+	+	×	—	—	+2	+	+
•Salmonella = 6 7 z_{4} 1 7	+	+	—	+	+	+	++	+	+	×	—	—	+2	+	+
S *stourbridge* = 6 8 b 1 6	+	+		+	+	+	++	+	—	+	×	—	+	+	—
S sterrenbos = 6 8 d e n x	+	+	—	+	+	+	++	+	—	+	+	—	+	+	—
S eimsbuttel = 6 (7) (14) d 1 w	+	+	—	+	—	+	++	+	—	—	+	+	+	+	—
S bornum = 6 (7) (14) z_{38}	+	+		+	+	+	++	+	—	+	+	—	+	+	—
•S manica = 1 9 12 g m s t z_{4}	+	+	+	+	+	+	++	+	+	×	—	—	+2	+	+
S new mexico = 9 12 g (p) 1 5	+	+	d	+	—	+	++	+	+	+	—	—	+	[illegible]	—
S lawndale = 1 9 12 z 1 5	+	+	—	+	+	+	++	+	—	+	+	—	+2	—	—
S *stellenbosch* = 1 9 12 z 1 7	+			+	+	+	++	+	+	—	—	—	+	—	+
S mathura = (9) 46 i e n z_{15}	+	+	—	—	+	+	—	—	—	+	×	+	+	+	—
S cevco = (9) 46 k z_{35}	+	+	+	+	+	+	++	+	—	+	×	—	+	+	—
S pramiso = 3 10 c 1 7	+	+	+	+	+	+	++	+	—	+	+	+2	+	+	—
•S fuhlsbuttel = 3 10 l v z_6	+	+		+	+	+	++	+	+	×	—	—	+2	+	—
S *ruzizi* = 3 10 l v e n z_{15}	+	+	—	+	—	+	++	+	—	+	+	—	+	+	—
S fallowfield = 3 10 l z_{13} z_{28} e n z_{15}	+	+	+	+	+	+	++	+	—	—	+	—	+	+	—
•S winchester = 3 10 z_{39} 1 7	+	+	—	+	+	+	++	+	+	—	—	—	+2	+	+
S rosenthal = 3 15 b 1 5	+	+	+	+	+	+	++	+	—	+	×	—	+	+	—
S *drypool* = 3 15 g m s	+	+	+	+	+	+	++	+	—	+	+2	+3	+	+	—
S tilburg = 1,3 19 d 1 w	+	+	+	+	+	+	++	+	—	+	+	+	+	+	—
S nyanza = 11 z z_6	+	+	+	+	+	+	++	+	—	+	×	—	+	+	—
S straengnaes = 11 z_{10} 1,5	+	+	—	+	+	+	++	+	—	+	+	+2	+	+	—
Salmonella = 13 22 b e n z_{15}	+	+		+	+	+	++	+	—	+	+	+3	+	+	—
S ferlac = 1 6 14 25 a e n x	+	+	—	+	+	+	++	+	+	×	—	+	+	+	—
•S bellville = 16 e n x 1 7	+	+	—	+	+	+	++	+	+	—	—	—	+2	+2	+
S frankfurt = 16 i e n z_{15}	+	+		+	+	+	++	+	—	+	×	—	+	+	—
•Salmonella 16 z_{42} 1 (5) 7	+	+	—	+	+	+	++	+	+	×	—	—	+	+	+
S langenhorn = 18 m t	+	+		+	+	+	++	+		+	×	—	+2	+	
S gambaga = 21 z_{35} e n z_{15}	+	+		+	+	+	++	+		+	×		+		

S ashanti — 28 b 1 6					+	+	++				+		$+^{2}$	+	
S hermannswerder — 28 c 1 5	+	+			+	+	++	+		+	×		+	+	
Salmonella — 28 l e n z15		+		+	+	+	++	+		+	×		+	+	
S babelsberg — 28 z4 z23 e n z15		+		+	+	+	++	+		+	×		$+^{2}$	+	
Salmonella — 28 z10 e n z15	+	+		+	+	+	++	+		+	×		+	+	—
S wayne — 30 g (p)	+	+		+	+	+	++	+	+	+			$+^{2}$	+	—
S kumasi — 30 z10 e n z15	+	+	$+^{6}$	+	+	+	++	+		+	+		+	+	+
Salmonella — 35 z4 z23	+	+		+	+	+	++	+		+	+	$+^{2}$	+	+	—
*S [illegible] — 39 l z28 e n x	+	+		+	+	+	++		+		—		$+^{2}$	$+^{1\,2}$	+
S anfo — 39 y 1,2	+	+	+	+	+	+	++	+		+	+	$+^{2}$	+	+	—
S driffield — 1 40 d 1 5	+	+	+	+	+	+	++	+	—	+	×	—	+	+	—
S tilene — 1 40 e h 1 2	+	+		+	+	+	++	+		+	+	+	+	+	—
S bern — 1 40 z4 z32	+			+	+	+		+	+	—	—			—	—
*Salmonella — 41 z10 z6	+	$+^{3}$		+	+	+	++	+	+	×	—		$+^{2}$	$+^{1\,2}$	+
S maricopa — 1 42 g (p) 1 5	+	+		+	+	+	++	+	+	+	—		$+^{2}$	$+^{1\,2}$	—
*S bunnik — 43 z42	+	+		+	+	+	++	+	+	—			×	+	—
S saka — 47 b	+	+		+	+	+	++	+		+	×	—	+	+	—
*S phoenix — 47 b 1 5	+	+		+	+	+	++	+	+			—	$+^{2}$	+	+
S bootle — 47 k 1 5	+	+	—	+	+	+	++	+	—	+	×	$+^{2}$	+	+	—
S mount pleasant — 47 z 1 5	+	+		+	+	+	++	+		+	—		+	+	—
*S sakaraha — 48 k z39	+	+		+	+	+	++	+	+	—	—	—	$+^{2}$	+	+
S overschie — 51 l v 1 5	+	+		+	+	+	++	+	—	+	×		+	+	—
S flottbek — 52 b	+	+		+	+	+	++	+	—	+	×	—	$+^{2}$	+	—
*S midhurst — 53 l z28 z39	+	+		+	+	+	++	+	+	×	—	—	$+^{2}$	+	+

Key Ara — arabinose Dul — dulcitol Ino — inositol Rha — rhamnose Tre — trehalose Xyl — xylose Gly — Stern's glycerol fuchsin broth Gel — gelatin d — d tartrate l — l tartrate i — i tartrate Cit — sodium citrate Muc — mucate Mal — sodium malonate

Arabinose to xylose + — positive after 1 day
$+^{1\,2}$ — positive after 1–2 days
— negative after 30 days
× — late and irregularly positive or negative
d — different biochemical types

Stern's glycerol fuchsin broth ++ lilac after 1 2 days
negative after 8 days

Gelatin + — slowly positive in ferrous chloride gelatin and rapidly positive in the Kohn test modified by Lautrop
Organic acids + — positive after 1 day $+^{2\,3}$ — positive after 2 3 days - — negative after 14 days * — sub genus II

List of Salmonella Species Recognized in 1960

Salmonella alamo Edwards, Kauffmann & Fife = 6,7 g,(p) 1,5
Acta path et microbiol scandinav in press
Salmonella albert = 4,12 z_{10} e,n,x
Salmonella anfo = 39 y 1,2
Salmonella ashanti = 28 b 1,6
Salmonella babelsberg Hofmann = 28 z_4,z_{23} e,n,z_{15}
Zbl Bakter I Orig in press
Salmonella bellville = 16 e,n,x 1,7
Salmonella bern Kauffmann, Fey & Steck = 1,40 z_4,z_{32} –
Acta path et microbiol scandinav , 50, 335, 1960
Salmonella bornum Hofmann, Kauffmann & Anz = 6,(7),(14) z_{38}
Zbl Bakter I Orig *179*, 39, 1960
Salmonella bottle = 47 k 1,5
Salmonella bunnik = 43 z_{42} –
Salmonella ceyco = (9),46 k z_{35}
Salmonella coleypark = 6,7 a l,w
Salmonella driffield = 1,40 d 1 5
Salmonella drypool = 3,15 g,m,s
Salmonella eimsbuettel Rohde & Bischoff = 6,(7),(14) d l,w
Zbl Bakter I Orig in press
Salmonella fallowfield = 3,10 l z_{13},z_{28} e,n,z_{15}
Salmonella ferlac = 1,6,14,25 a e,n,x
Salmonella flottbek Rohde = 52 b
Zbl Bakter I Orig in press
Salmonella frankfurt Hofmann = 16 i e,n,z_{15}
Zbl Bakter I Orig in press
Salmonella fuhlsbuettel Rohde, Ruchhoff & Tiedje = 3 10 l,v z_6
Zbl Bakter I Orig in press
Salmonella gambaga = 21 z_{35} e n,z_1
Salmonella gdansk nom nov *Kauffmann* = 6 7 l v z_6
(*Salmonella gdansk* *Buczowski* Bull Inst Mar Med Gdansk *11*, 59, 1960)
Salmonella hermannswerder Hofmann = 28 c 1,5
Zbl Bakter I Orig in press
Salmonella kumasi = 30 z_{10} e n z_1
Salmonella langenhorn Rohde = 18 m,t
Zbl Bakter I Orig in press
Salmonella lawndale = 1 9 12 z 1,5
Salmonella manica = 1,9,12 g m s t z_{42}
Salmonella maricopa Edwards, Kauffmann & Fife = 1,42 g (p) 1,5
Acta path et microbiol scandinav in press
Salmonella mathura = (9),46 i e,n z_{15}
Salmonella midhurst = 53 l,z $_{28}$ z_{19}
Salmonella mondeor = 39 l,z_{28} e n,x

Salmonella mount pleasant = 4 7 z 1 5
Salmonella new mexico Edwards Kauffmann & Fife — 9 12 g (p) 1 5
Acta path et microbiol scandinav in press
Salmonella nyanza = 11 z z
Salmonella othmarschen Rohde = 6 7 g m t
Zbl Bakter I Orig in press
Salmonella overschie = 51 l v 1 5
Salmonella phoenix — 47 b 1 5
Salmonella pramiso — 3 10 c 1 7
Salmonella rosenthal Hofmann = 3 15 b 1 5
Zbl Bakter I Orig in press
Salmonella ruki nom nov *Kauffmann* = 4 5 12 y e n x
(Salmonella ruki *Vassiliadis Le Minor & Donkers* Bull Soc Path Exot in press)
Salmonella ruzizi nom nov *Kauffmann* — 3 10 l v e n,z_{15}
(Salmonella ruzizi *Vassiliadis van Ros & Herman* Bull Soc Path Exot in press)
S = 47 b
st Afric Med J *9*, 172 1960)

Salmonella stellenbosch — 1 9 12 z 1 7
Salmonella sterrenbos = 6 8 d e n x
Salmonella stourbridge = 6 8 b 1 6
Salmonella straengnaes Alin Backelin Bengtsson & Mattsson = 11 z_{10} 1 5
Acta path et microbiol scandinav in press
Salmonella tilburg = 1 3 19 d l w
Salmonella tilene = 1 40 e h 1 2
Salmonella wayne Edwards Kauffmann & Fife = 30 g (p)
Acta path et microbiol scandinav in press
Salmonella wilhelmsburg Rohde & Bischoff — 4 12 27 z_{38} —
Zbl Bakter I Orig in press
Salmonella winchester = 3 10 z_{39} 1 7
Salmonella ? = 4 12 b 1 6
Salmonella ? — 6 7 z_{39} 1 7
Salmonella ? 6 7 z_{15} 1 7
Salmonella ? = 13 22 b e n,z_{15}
Salmonella ? — 16 z_{10} 1 (5) 7
Salmonella ? 28 i e n,z_{15}
Salmonella ? = 28 z_{10} e n,z_{15}
Salmonella ? — 35 z_4,z_{24}
Salmonella ? — 41 z_{10} z_6

Addendum to Supplement III
(Kauffmann 4)

Salmonella remo = 1,4,12,27: r: 1,7
Salmonella heilbron = 6,7: l,z_{28}: 1,5
Salmonella kuru = 6,8: z: l,w
Salmonella stendal = 11: l,v: 1,2
Salmonella bokanjac = 28: b: 1,7
Salmonella greiz = 40: a: z_6
Salmonella leipzig = 41: z_{10}: 1,5

SUMMARY

A supplement to the review *"Das Kauffmann-White-Schema"* is given, containing 62 new *Salmonella-species* recognized during the year 1960.

REFERENCES

Kauffmann, F. (1) "Das Kauffmann-White Schema" in Ergebnisse der Mikrobiologie Immunitätsforschung und Experimentellen Therapie *30* 160, 1957 (Springer Verlag, Berlin-Gottingen-Heidelberg)
Kauffmann, F. (2) "Two biochemical sub divisions of the genus Salmonella" Acta path et microbiol scandinav *49* 393 1960
Kauffmann F (3) "The species definition in the family Enterobacteriaceae" International Bull Bacter Nomen & Taxon *11* 5, 1961
Kauffmann, F (4) "Supplement to the Kauffmann-White Schema (III)" Acta path et microbiol scandinav *49* 89 1960

FROM THE SERUM AND TOXOID DEPARTMENT AND THE EPIDEMIOLOGICAL DEPARTMENT, STATENS SERUMINSTITUT, COPENHAGEN

SIMULTANEOUS TETANUS IMMUNIZATION AND REINFORCEMENT OF DIPHTHERIA IMMUNITY IN ADULTS, WITH SPECIAL REFERENCE TO THE INTERFERENCE PHENOMENON

By

INGA SCHEIBEL and SVEND TULINIUS

Received 24.xii.60

During World War II there was a considerable increase in the incidence of diphtheria in Denmark.

Extensive diphtheria immunization was undertaken at that time, and a very high percentage—more than 90 per cent—of persons below the age of 18 were immunized.

From 1950 the combined diphtheria-tetanus vaccine replaced the monovalent diphtheria vaccine.

When tetanus immunization was introduced as a general prophylactic measure in the army, it was considered desirable to follow up the diphtheria immunization by using the combined vaccine for all three injections on those who, for some reason or other, had not previously been immunized against diphtheria, and to reinforce the diphtheria immunity in those who had been immunized in childhood by using a combined vaccine for at least one of the three injections.

The question of possible interference from previous diphtheria immunization with the response to the tetanus component in the combined vaccine then arose.

In 1953 and 1955 *Barr & Llewellyn-Jones* (1, 2, 3) published a series of experiments showing a deteriorating effect from pre-existing immu-

This study would not have been possible without the ready cooperation of the staff of the Army [illegible]

We are especially indebted to *Colonel Boy Kromann* M.C. for his invaluable help throughout the whole field work.

The best thanks of the writers are due to Dr. *Flo Christensen* of the Serum and Toxoid Department, Statens Seruminstitut, who has given valuable help whenever requested, and to Mrs. *Aarkrogh* and Miss *Steenberg* for their excellent technical assistance.

The statistical work has been carried out by the Statistical Department of the Statens Seruminstitut, chief *Michael Weis Bentzon*, Actuary.

nity to one of the antigens in a combined vaccine on the response to the *other antigen(s) in the vaccine new to the organism* These results have to a certain degree been confirmed in children by *Chen et al* (4,5)

From a theoretical point of view, this is an interesting observation *and such interference might become a problem of importance to the* health authorities as the use of combined vaccines for routine immunization and reinforcement of immunity increases

In order to further elucidate the problem and obtain information as to the most adequate way of instituting primary immunization against tetanus together with a reinforcement of the diphtheria immunity in adults *immunized against diphtheria in childhood, the following in*vestigation was undertaken

MATERIAL AND METHODS

724 persons of the army personnel 18–28 years of age who entered military service in May July and September 1957 belonging to two categories of the army one a *general infantry regiment the other the Royal Danish Life Guards* were divided into four different groups and immunized according to the plan given in Table 1

TABLE 1

Group	1st injection	2nd injection 4 weeks later	3rd injection 1 year later
A	D + T*	D + T	D + T
B	T §	T	T
C	D + T	T	T
D	T	D + T	T

* D + T = combined diphtheria tetanus vaccine
§ T = tetanus vaccine

The grouping was made completely at random by allocating the men to each group alternately as they reported for the first injection

Vaccines The vaccines used consisted of purified diphtheria and/or purified tetanus toxoid adsorbed on to $Al(OH)_3$ The concentration in flocculation units was about 12 per ml for both antigens both as regards the combined vaccine and the tetanus vaccine The concentration *of $Al(OH)_3$ corresponded to 1 mg of Al per ml* vaccine

Two different batches of both diphtheria and tetanus toxoids were employed When assayed in guinea pigs as a combined diphtheria tetanus vaccine the two *diphtheria toxoids were of the same antigenic potency* whilst the two tetanus toxoids were of significantly different antigenic potency both when assayed alone and in combination with diphtheria toxoid Estimation of the antigenic potency was carried out as described in (10)

The purity of the toxoids and the antigenic potencies of the adsorbed vaccines can be seen from Table 2

Assay of antitoxin Titration for tetanus antitoxin was undertaken by means of *Ipsen's* method (7)

Titration for diphtheria antitoxin was carried out by haemagglutination (12) and the titers expressed *in international units using the international standard for* diphtheria antitoxin as reference (9)

It became apparent during the investigation that some persons possessed neutralizing antitoxin which did not agglutinate [1] All pre immunization sera and some

[1] This problem will be dealt with in a later publication

of the samples taken later were therefore retested by means of the intracutaneous test (8)

In no instance was it seen that a serum had a positive haemagglutination titer without containing antitoxin

TABLE 2

Purity of the Toxoids and Antigenic Potency of the Vaccines

	Lf mg PN	Lf mg TN	Antigenic potency (in units ml)
Diphtheria toxoid I	1800	1600	65
Tetanus toxoid I	1800	1500	250
Diphtheria toxoid II	2050	2000	65
Tetanus toxoid II	850	800	100

Immunization and blood sampling The immunization was started at three different times during the summer 1957 May July and September The May and the September immunizations were performed with vaccines containing toxoids No 1 and the July immunization with vaccines containing toxoids No 2

In all cases the dose of vaccine was 1 ml given subcutaneously in the intraclavicular region

Blood sampling was carried out prior to the first injection prior to and two weeks after the second injection and prior to and two weeks after the third injection

State of immunity prior to immunization All persons known to have been immunized against tetanus or whose serum prior to the first injection was found to contain tetanus antitoxin were excluded from the experiment

Only those who received all three injections and from whom all blood samples were taken at the right time were included in the final calculations and comparisons This [illegible]

[illegible] since they were too few in number to influence the final results it was considered unnecessary *to re-calculate the whole material*

Calculations The antitoxic responses are calculated as the geometric means of the individual titers *The relative* frequencies of persons responding with less than 0 001 units of tetanus antitoxin per ml of the first injection of vaccine [illegible] 0 01 units per [illegible]

Means and [illegible]

the [illegible]

In testing the significance the differences between averages of the logarithmic titers were measured by their *standard errors* Thus for example, in comparing groups A and B after the first injection (Table 3) the difference was 2 58 (—3 20) = 0 62 with a standard error of $\sqrt{1.17^2/[illegible] + 1.22^2/[illegible]} = 0.18$ the standard deviations of the logarithmic titers being 1 17 and 1 22 The ratio 0 62 0 18 = 3 4 exceeds the value 1 96 corresponding to the five per cent limit *of significance*

TA[illegible]

Tetanus Antitoxin Response in Diphtheria Immune Adults to Immun[illegible]

TEAM I

Group	Number	1st inj	4 weeks after 1st inj			2nd inj	2 weeks a[illegible]	
			U ml	S D (log tit)	< 0 001 ml *, %		U ml	S D (log tit)
A	100	D + T	0 0026	1 17	42	D + T	0 19	0 58
B	73	T	0 0006	1 22	66	T	0 25	0 68
C	83	D + T	0 0039	1 17	31	T	0 41	0 67
D	90	T	0 00081	1 20	63	D + T	0 31	0 65

* As regards some persons the taking of the blood specimens after the first injecti[illegible] influenza epidemic This did not influence the results after the first injection significantly They have therefore been excluded from the calculations in this column the actua[illegible]

Vaccines I were, as mentioned given to the May and September categories These hereafter designated team I include a total of 346 with 100 persons in group A 73 in group B 83 in group C, and 90 in group D

The July categories given vaccines II comprise a total of 158 with 36 in group A 39 in group B, 38 in group C and 45 in group D These are designated team II

RESULTS

Tetanus antitoxin responses The results of the tetanus immunization of teams I and II are shown in Tables 3 and 4 respectively

As already mentioned, none of the persons included in the material had measurable tetanus antitoxin prior to the vaccination (i e < 0 0001 unit per ml), whilst all except three (see p 229) had immunity against diphtheria

The means of the titers of diphtheria antitoxin prior to first injection were alike for teams I and II, being approximately 0 08 units per ml with group variation between 0 06 and 0 11 (Tables 5 and 6)

Team I After the first injection, which was the same for groups A + C and B + D, the tetanus antitoxin response of team I was highest in the groups immunized with the combined vaccine The differences are highly significant, also when calculated for the groups separately The probability that they may due to chance is less than 0 1 per cent for group A versus group B, group C versus group B or group D, and between 0 1 and 0 5 per cent for group A versus group D

The means of the groups who received the same vaccines do not differ significantly

The relative number of persons responding with less than 0 001 units per ml to the first injection is likewise significantly smaller after immunization with D + T vaccine than with T vaccine, whilst the frequencies for groups receiving the same vaccine do not show differences larger than might be due to chance

3

with Combined Diphtheria Tetanus Vaccine and/or Tetanus Vaccine

TEAM I

2nd inj *	One year after 2nd inj			3rd inj	2 weeks after 3rd inj		
< 0.01 ml %	L ml	S D (log t t)	< 0.01 ml %		L ml	S D (log t t)	< 0.01 ml %
0	0.038	0.68	22	D + T	8.3	0.37	0
1.4	0.019	0.82	30	T	9.8	0.55	0
0	0.049	0.77	17	T	9.5	0.41	0
0	0.059	0.81	13	T	7.1	0.44	0

and the giving of the second injection had to be postponed for two weeks because of an whereas the titers after the second injection were significantly higher in these persons numbers of persons here being 75, 52, 63 and 6 for groups A to D respectively

These results show that no interference with the response to the tetanus toxoid had occurred after the first injection. On the contrary the combined vaccine showed a synergic effect.

Two weeks after the second injection, group A, which received the *combined vaccine only*, and group B, which received tetanus vaccine only, responded equally well, their mean titers not differing significantly.

Group C, which was given combined vaccine for the first injection and tetanus vaccine for the second, and group D, also given tetanus vaccine and combined vaccine but in reverse order, likewise differed only insignificantly in their mean titers.

The same applies to group B as compared to groups C and D, whereas the two last groups both had significantly higher mean titers than group A, P being 0.1 per cent for A versus C and 3 for A versus D. Interference with the tetanus response thus seems to have occurred in group A, although it is not demonstrable when comparing group A with group B.

It will be seen that out of the 346 persons in team I all but one—in the group given only T vaccine—now had more than 0.01 units per ml.

A year later the mean titers as usually seen showed an approximately tenfold decrease. At this time group B had a significantly smaller mean than any of the other groups, where the means show only chance variations. The interference observed in group A two weeks after the second injection is no longer seen.

The relative numbers of persons whose titer had dropped below 0.01 units per ml, which is ordinarily considered the protective level, vary between 13 and 30 per cent. This parameter reflects the same group differences as the mean titers, group B having the highest percentage of persons with no or poor protection. The difference between groups B

T4

Tetanus Antitoxin Response in Diphtheria Immune Adults to Immuni at

TEAM II

Group	Number	1st inj	4 weeks after 1st inj			2nd inj	2 weeks afte	
			U ml	S D (log ut)	< 0 001 ml *%		U ml	S D (log ut)
A	36	D + T	0 0037	1 29	36	D + T	0 24	0 72
B	39	T	0 0029	1 47	49	T	0 17	0 99
C	38	D + T	0 011	1 66	29	T	0 33	0 77
D	45	T	0 00087	1 17	53	D + T	0 28	0 65

and D is significant, whereas the other groups do not differ more than may be due to chance

The third and last injection provoked strong production of tetanus antitoxin

Groups B, C and D received tetanus vaccine for this injection, whilst *group A was given the combined vaccine Neither interference nor synergic* effect occurred, the means of the four groups varying from 7 1 for group D to 9 8 for group B 336 of 346 persons immunized now had more than 1 unit per ml, and the lowest individual titer observed was 0 28 units per ml

Team II The results for team II are presented in Table 4

As already mentioned, the vaccines used for this team differed from those used for team I, in that tetanus toxoid II was of significantly smaller antigenic potency than tetanus toxoid I, whilst the diphtheria toxoids I and II had the same antigenic potency It was considered that a possible interference might show more clearly when a tetanus vaccine of lower antigenic potency was applied However, it appeared that the difference in antigenic potency between the two tetanus toxoids found in the animals was not observed in human beings and thus the basis for this part of the study did not materialize

After the first injection the trend is the same as for team I, the means for the groups which received tetanus vaccine being numerically smaller than those for the groups given combined vaccine The differences observed are statistically significant only between groups A and D and between groups C and D However, when the calculation is carried out on A + C versus B + D the difference becomes highly significant, P being between 0 1 and 1 per cent The same applies to the relative numbers of persons responding with less than 0 001 units per ml

No significant differences can be shown between similarly treated groups either as regards the means or the percentages of persons who responded poorly

After two injections, groups A and B had the numerically lowest

h Combined Diphtheria-Tetanus Vaccine and/or Tetanus Vaccine

TEAM II

inj	One year after 2nd inj			3rd inj	2 weeks after 3rd inj		
< 0.01 ml %	t ml	S D (log tit)	< 0.01 ml %		t ml	S D (log tit)	< 0.01 ml %
0	0.093	0.41	3	D + T	12.5	0.47	0
10	0.059	0.95	23	T	10.2	0.50	0
3	0.076	1.02	16	T	11.0	0.43	0
0	0.056	0.82	22	T	10.7	0.46	0

means and group C the highest, as was also the case for team I. For team II, however, the differences observed are not significant. In group B 10 per cent and in group C 3 per cent had titers below 0.01 units per ml. All (5 persons) had produced antitoxin, having more than 0.001 units per ml.

A year later the usual drop in the mean titers was seen. No significant differences could be shown between the groups.

As regards persons with titers below the protective level, groups B and D had significantly higher percentages than group A, whilst the other groups did not differ significantly.

Two weeks after the third injection all mean titers had increased to more than 10 units per ml. No significant difference can be shown between the groups. 156 out of the 158 persons had titers higher than one unit per ml. The lowest individual titer observed was 0.6 units per ml.

Variability in response. The decreasing variability of the individual responses generally observed during the course of an immunization is also apparent in the present material. From Tables 3 and 4 it is evident that the standard deviations are highest after the first injection, show a considerable decrease after the second injection, increase somewhat during the following year, and finally reach their minimum value after the third injection. There are no significant differences between the standard deviation within the four groups, except as regards team II after the second injection, where group A, for unknown reasons, has a significantly smaller standard deviation than any of the other three groups.

Diphtheria antitoxin response. The response to the diphtheria component in the combined vaccine is presented in Tables 5 and 6.

As mentioned earlier, some sera contained neutralizing antitoxin which did not haemagglutinate, and some contained a mixture of both kinds of antitoxin molecules. For the sake of comparison, only the haemagglutination titers have been used for calculating the means given

TABL[illegible]

Tetanus Antitoxin Response in Diphtheria Immune Adults to Immuni at[illegible]

TEAM II

Group	Number	1st inj	4 weeks after 1st inj			2nd inj	2 weeks [illegible]	
			U ml	S D (log tit)	< 0 001 ml *,		U ml	S D (log t[illegible])
A	36	D + T	0 0037	1 29	36	D + T	0 24	0 72
B	39	T	0 0029	1 47	49	T	0 17	0 99
C	38	D + T	0 011	1 66	29	T	0 33	0 77
D	45	T	0 00087	1 17	53	D + T	0 28	0 65

and D is significant, whereas the other groups do not differ more than may be due to chance

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No significant differences can be shown between similarly treated groups either as regards the means or the percentages of persons who responded poorly

After two injections, groups A and B had the numerically lowest

...h Combined Diphtheria Tetanus Vaccine and/or Tetanus Vaccine

TEAM II

... inj	One year after 2nd inj			3rd inj	2 weeks after 3rd inj		
< 0.01 ml %	U ml	S D (log tit)	< 0.01 ml %		U ml	S D (log tit)	< 0.01 ml %
0	0.093	0.41	3	D + T	12.5	0.47	0
10	0.059	0.95	23	T	10.2	0.50	0
3	0.076	1.02	16	T	11.0	0.43	0
0	0.056	0.82	22	T	10.7	0.46	0

means and group C the highest, as was also the case for team I. For team II, however, the differences observed are not significant. In group B 10 per cent and in group C 3 per cent had titers below 0.01 units per ml. All (5 persons) had produced antitoxin, having more than 0.001 units per ml.

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As mentioned earlier, some sera contained neutralizing antitoxin which did not haemagglutinate, and some contained a mixture of both kinds of antitoxin molecules. For the sake of comparison, only the haemagglutination titers have been used for calculating the means given

TABLE 5

Diphtheria Antitoxin Response in Adults to Re Immunization with Combined Diphtheria Tetanus Vaccine 10 16 Years after Diphtheria Immunization

Group	Pre immunization U ml	1st inj	4 weeks after 1st inj U ml	2nd inj	2 weeks after 2nd inj U ml	One year after 2nd inj U ml	3rd inj	2 weeks after 3rd inj U ml
A	0 06 (97)	D + T	6 9 (100)	D + T	8 7 (75)	1 12 (100)	D + T	3 64 (100)
B	0 11 (73)	T	not measured	T	not measured	0 08 (71)	T	not measured
C	0 11 (83)	D + T	10 02 (82)	T	not measured	1 25 (83)	T	not measured
D	0 07 (90)	T	0 08 (87)	D + T	7 0 (65)	1 10 (90)	T	not measured

Figures in bracket indicate the number of persons

TABLE 6

Diphtheria Antitoxin Response in Adults to Re Immunization with Combined Diphtheria Tetanus Vaccine 10–16 Years after Diphtheria Immunization

Group	Pre immunization U ml	1st inj	4 weeks after 1st inj U ml	2nd inj	2 weeks after 2nd inj U ml	One year after 2nd inj U ml	3rd inj	2 weeks after 3rd inj U ml
A	0 08 (36)	D + T	7 4 (36)	D + T	7 8 (36)	1 20 (36)	D + T	3 8 (36)
B	0 06 (39)	T	0 09 (38)	T	0 06 (38)	0 05 (37)	T	0 06 (37)
C	0 09 (38)	D + T	7 6 (38)	T	4 5 (38)	1 0 (38)	T	1 25 (38)
D	0 08 (45)	T	0 08 (45)	D + T	6 6 (45)	0 83 (45)	T	0 98 (45)

Figures in bracket indicate the number of persons

below These are somewhat lower than would have been the case had the intracutaneous method been used throughout A provisional calculation indicates that the intracutaneous method gives mean values twice as high as those found by the haemagglutination method

A more detailed description of the duration of the diphtheria immunity will be published separately In the present work attention will be drawn only to a few points of importance for the problems under discussion here

There was not enough serum in all cases to carry out assessment of both diphtheria and tetanus antitoxin The number of persons therefore varies slightly and is given in brackets after the corresponding mean titer The smaller number of persons in groups A and D team I two weeks after the second injection is due to delayed blood sampling from one of the categories at that time (see p 230 31)

The figures show that all groups given D + T vaccine responded equally well to the diphtheria antigen the differences of the means being no bigger than expected from chance variations Secondary diphtheria antitoxin production is thus independent of whether a simultaneous production of tetanus antitoxin is of primary or secondary nature

In 1953 *Slavitsky* (11) reported that injection of tetanus toxoid into rabbits previously immunized against diphtheria and tetanus resulted in the appearance of both diphtheria antitoxin and tetanus antitoxin in the serum The B groups in the present investigation show clearly that a non specific anamnestic diphtheria antitoxin response does not occur in human beings

Reactions to the injections There were very few untoward reactions as the result of the injections Twenty eight persons or 3 9 per cent of the total material (24 persons) reported some local reaction consisting of a little swelling redness and soreness at the site of injection These reactions which were of no real importance were reported both after the D + T vaccine and after the T vaccine and both after the first and after the following injections

Four persons (0 5 per cent) had a general reaction Two of these were very slight the persons only feeling unfit for about one day without fever or any other well defined symptoms One of these reactions occurred after the T vaccine and one after the combined vaccine The two other general reactions which both occurred in persons given D + T vaccine were rather pronounced with fever up to 40° C one of the persons had to stay in bed for seven days and the other for two days

DISCUSSION

After the first injection of group A and C (D T accine) in the toxoid during

The ratio of produced circulating diphtheria antitoxin to that of tetanus antitoxin when measured after four weeks is rather remarkable. A calculation on the assumption that the two antitoxin molecules have a molecular weight of approximately the same order shows that *about 2000 times as much diphtheria* antitoxin as tetanus antitoxin was produced after the first injection. That this is possible without interference with the tetanus response indicates that the two antitoxins are produced by mechanisms which work completely independently. *Barr & Llewellyn-Jones* (1) made a similar observation in guinea pigs given one injection, whereas *Chen et al.* (4) report interference in children also after the first injection. In both these cases, however, the control groups did not, as in our investigation, consist of immune individuals injected only with one antigen new to the organism but of non-immune individuals injected with the combined antigens.

At the time of the second injection, the tetanus antitoxin producing mechanism had been activated; this is clearly demonstrated by a 70–400 fold increase in circulating tetanus antitoxin in the following two weeks. Thus the antitoxin production in function in the D groups after this injection is a simultaneous secondary response to two different antigens. By comparing the tetanus response in these groups to that in the groups which received only T vaccine for the second injection and the diphtheria response to that in the A groups after the first injection, it can be seen that simultaneous secondary response to two antigens has taken place without interference, as was also the case with simultaneous primary and secondary responses. In the A group of team I, however, interference is demonstrable when the means of tetanus antitoxin in this group are compared with those in group C and D, but not when compared with group B. The A group in team II shows the same trend but here without significant manifestation. This interference is not easy to understand, as little or no production of diphtheria antitoxin had taken place in the A groups after the second injection.

At the time of this injection group A differed from group D in having a high concentration of circulating diphtheria antitoxin prior to the injection, and from group C in having been given both diphtheria and tetanus toxoid.

The C groups show that the interference in the A group cannot be explained either through a possible exhaustion of the antibody producing system caused by its recent secondary activity or by a prolonged secondary response, as suggested by *Barr & Llewellyn-Jones* (2).

Interference has been observed by one of the writers (*Scheibel*, unpublished) in guinea pigs under similar experimental conditions. The material consisted of three groups of guinea pigs immunized four weeks previously with varying doses of diphtheria vaccine and consequently containing varying concentrations of circulating diphtheria antitoxin at the time of the experiment. The control groups were non-immune animals. All immunizations were carried out with $Al(OH)_3$-

adsorbed D + T vaccine The only group where interference occurred after the first injection was that with the highest pre existing concentration of circulating diphtheria antitoxin (approx 1 unit per ml) In the two other groups with 0 06 and ≤ 0 001 units per ml of pre existing diphtheria antitoxin no interference occurred After two injections interference could also be shown in the group with 0 06 units per ml of diphtheria antitoxin prior to the first injection This group had a mean of about 8 units per ml when given the second injection In the third group with about 0 1 units per ml of diphtheria antitoxin prior to the second injection no interference could be demonstrated

Chen et al from their study in children (4) conclude that the degree of interference intensifies with increasing pre existing diphtheria immunity and state that a diphtheria antitoxin level of 0 01 u ml represents that degree of immunity over and under which the intensity of interference differs significantly Although the level found by those authors is much lower than that at which interference occurred in the present studies in human adults and in guinea pigs their observation conforms with ours in principle and there seems to be a connection between the degree of pre existing immunity and the interference phenomenon Attempts to investigate a possible correlation further were made by plotting the individual titers of tetanus antitoxin after the first and second injection against the corresponding titers of pre-existing diphtheria antitoxin for each group However this did not give any real additional information

At a certain concentration of circulating antitoxin re injection of the corresponding antigen will lead to the formation of antigen antibody complexes The present results indicate that these complexes or perhaps the antigen antibody reaction as such play a role since the interference cannot be explained either by simultaneous production of two different antitoxins or by recent high activity of the antibody producing system In this connection attention should be drawn to a recent work by *Hayashi et al* (6) and *Tokuda et al* (13) which shows that application of the corresponding antigen to cultures of living sensitized monocytes causes rapid release of a protease with pH optimum 7 4 into the extracellular (chamber [illegible] the release of inhibitor against this [illegible] is at work in human beings this ma[illegible] interference

The hypothesis given above is contradicted by the fact that group A responds just as well as group B It is a possibility that the synergic effect of the combined vaccine which became clearly evident after the first injection has obscured an interference phenomenon

The interference observed after the second injection could not be demonstrated in connection with the third injection The level of diphtheria antitoxin in group A prior to this injection was about one unit [illegible] [illegible]es less than prior to the second injection Contra[illegible] [illegible]pened after the second injection the A group pro

duced diphtheria antitoxin on the third injection, although the mean titers did not reach the same value as after the first booster dose The fact that interference could not be shown on this occasion again seems to contradict the suggestion that antigen-antitoxin complexes or their formation may be the interfering principle but quantitative differences may be responsible for this

The results of the present study do not conform very well with those of *Barr et al* in guinea pigs (2, 3) or of *Chen et al* in children (4, 5) However, the experimental conditions have differed a great deal

The vaccines employed have varied somewhat, both in their absolute and relative concentrations of flocculation units Lack of information renders comparison of their antigenic potency impossible

The degree of pre-existing immunity and the time interval from its establishment also differed

The differences as regards the control groups have already been mentioned However, in the experiment described by *Barr & Llewellyn-Jones* in 1953 (2) there are groups of animals corresponding in principle to all four groups in the present study Those authors measured only the response to the second injection, and contrary to the present findings they observed a weaker response to the tetanus toxoid in the groups corresponding to our A and C groups than in the groups corresponding to our B and D groups No satisfactory explanation for the discrepancies can so far be offered

It would seem, however, that if interference had been a fundamental factor in simultaneous immunization of human beings, it should have disclosed itself clearly in our investigations

CONCLUSION

From a practical point of view, the transient interference observed in the present study is of no consequence The inference to be drawn is that in adults with pre-existing diphtheria immunity, tetanus immunization may safely be carried out at the same time as re-inforcement of the diphtheria immunity by using a combined D + T vaccine, at least if this is similar in composition and antigenic potency to the one used in the present study It does not matter whether the combined vaccine is administered as the first or second injection Routine vaccination against diphtheria and tetanus may also be carried out safely with such a vaccine, regardless of the state of diphtheria immunity of the individuals in the population

SUMMARY

346 adults immunized against diphtheria in childhood and still immune to diphtheria were immunized against tetanus in four different ways, by

(A) Three injections of combined diphtheria tetanus vaccine
(B) Three injections of tetanus vaccine
(C) One injection of combined vaccine and two of tetanus vaccine
(D) One injection of tetanus vaccine one of combined vaccine and one of tetanus vaccine

After the first injection no interference with the tetanus response but a synergic effect from the combined vaccine had occurred After the second injection some interference was demonstrable in group A as compared with groups C and D but not as compared with group B. These three latter groups did not differ significantly in their response to the tetanus toxoid The possible reasons for this are discussed After the third injection neither interference nor synergic effect were seen

Another similar group of 158 adults was immunized in the same way, but with a tetanus toxoid of significantly lower antigenic potency when tested in the laboratory The interference seen in group A in the first team did not appear to any significant extent in the second team, although the trend was the same

Under the conditions of this study, the interference observed has no practical consequences

REFERENCES

1 *Barr M & Llewellyn-Jones M* Some factors influencing the response of animals to immunisation with combined prophylactics Brit J Exper Path 34 12 1953

2 *Barr M & Llewellyn-Jones M* Interference with antitoxic responses in immunisation with combined prophylactics Brit J Exper Path 34 233 1953

3 *Barr M & Llewellyn-Jones M* Some factors influencing the response to immunisation with single and combined prophylactics Brit J Exper Path 36 147 1955

4 *Chen B L Chou C T Huang C T Wang Y T Ko H H & Huang W C* Studies on diphtheria pertussis tetanus combined immunisation in children I Heterologous interference of pertussis agglutinin and tetanus antitoxin response by pre existing latent diphtheria immunity J Immunol 77 144 1956

5 *Chen B L Chou C T Huang C T Wang Y T Ko H H Huang W C & Ling C F* Studies on diphtheria pertussis tetanus combined immunisation in children II Immune responses after the primary vaccination J Immunol 79 39 1957

6 *Hayashi H Tokuda A & Udaka K* Biochemical study of [illegible] ant [illegible] J E [illegible]

7 *Ipsen J* [illegible] tox [illegible]

8 *Jensen C* [illegible] sca [illegible]

9 *Scheibel I* A comparative study on intracutaneo [illegible]

10 *Scheı* [illegible] its antigenic potency in international units Int Ass Soc Microbiol Proc Third Int Meeting of Biol Stand Opatija 2 6 Sept 1957

11 *Stavitsky A B* The influence of adrenal cortical extract on antibody formation including the nonspecific anamnestic reaction in the rabbit J Infect Dis *92* 130 1953
12 *Stavitsky A B* Micromethods for the study of proteins and antibodies I Procedure and general applications of hemagglutination and hemagglutination inhibition reactions with tannic acid and protein treated red blood cells J Immunol *72* 360 1954
13 *Tokuda A Hayashi H & Matsuba K* Biochemical study of cellular antigen antibody reaction in tissue culture II Release of a protease inhibitor J Exper Med *112* 249 1960

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STUDIES IN LABORATORY ESTIMATION OF RHEUMATOID ARTHRITIS SERUM FACTOR

4. The Role of Gamma Globulin and Albumin for the Acryl Particles Test for RAS Factor[1]

By

STEN WINBLAD

Received 2 : 61

Methods for demonstrating RAS factor use three essentially different technique. One of these techniques is based on the agglutination of homologous sensitized animal red blood cells, mainly sheep blood cells (*Waaler* 22, *Rose, Regan, Pearce & Lipman* 17). Rh+ blood cells sensitized with anti D serum can also be made to agglutinate provided no inhibitory serum factor of type Gm a+ is present (*Grubb* 11). The other and possibly essentially different technique is that using agglutination of non biological particles in a sensitizing environment of gamma globulin. Such particles are polystyrene latex (*Singer & Plotz* 21), which are most widely used, collodion particles (*Zavazal* 27), bentonite particles (*Bloch & Bunim* 1), acryl (polymethyl metaacryl plast particles) (*Winblad* 28), and mastix (*Pit* 17). A third method is that based on experience that gamma globulin, aggregated when heated at 63° C for 30 minutes (*Christian* 4) reacts with the RAS factor with sedimentation of precipitin (*Epstein, Engleman & Ross* 6, *Vaughan* 27, *Muller* 16, *Winblad* 29).

In the agglutination reaction using plastic particles the presence of gamma globulin as sensitizing (reactant) was believed to be necessary. This is probably the reason why such reactions were called fixation tests. Bovine gamma globulin can replace human gamma globulin in this reaction (*Burby & Behr* 3) as well as other animal gamma globulins provided that they are made poor in albumin (*Rheins, McCoy, Buehler & Burrell* 18). The patient's serum has its own inherent gamma globulin and the action of the gamma globulin was regarded as the cause of the agglutination of plastic particles even if no sensitizing extra gamma globulin was added (*Singer & Plotz* 24, *Rheins, McCoy & Wall* 19, *Winblad* 30).

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I have to thank Mrs. *Britt Beronius* for technical assistance.

11 *Stavitsky A B* The influence of adrenal cortical extract on antibody formation including the nonspecific anamnestic reaction in the rabbit J Infect Dis *92* 130 1953
12 *Stavitsky A B* Micromethods for the study of proteins and antibodies I Procedure and general applications of hemagglutination and hemagglutination inhibition reactions with tannic acid and protein treated red blood cells J Immunol 72 360 1954
13 *Tokuda A Hayashi H & Matsuba K* Biochemical study of cellular antigen antibody reaction in tissue culture II Release of a protease inhibitor J Exper Med *112* 249 1960

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STUDIES IN LABORATORY ESTIMATION OF RHEUMATOID ARTHRITIS SERUM FACTOR

4. *The Role of Gamma Globulin and Albumin for the Acryl Particles Test for RAS Factor*[1]

By

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Methods for demonstrating RAS factor use three essentially different technique. One of these techniques is based on the agglutination of homologous sensitized animal red blood cells, mainly sheep blood cells (*Waaler* 22, *Rose, Regan, Pearce* & *Lipman* 17). Rh+ blood cells sensitized with anti-D serum can also be made to agglutinate provided no inhibitory serum factor of type Gm a+ is present (*Grubb* 11). The other and possibly essentially different technique is that using agglutination of non-biological particles in a sensitizing environment of gamma globulin. Such particles are polystyrene latex (*Singer* & *Plotz* 21), which are most widely used, collodion particles (*Zavazal* 27), bentonite particles (*Bloch* & *Bunim* 1), acryl (polymethyl metaacryl plast particles) (*Winblad* 28), and mastix (*Pil* 17). A third method is that based on experience that gamma globulin, aggregated when heated at 63° C for 30 minutes (*Christion* 4) reacts with the RAS factor with sedimentation of precipitin (*Epstein, Engleman* & *Ross* 6, *Vaughan* 27, *Muller* 16, *Winblad* 29).

In the agglutination reaction using plastic particles the presence of gamma globulin as sensitizing (reactant) was believed to be necessary. This is probably the reason why such reactions were called fixation tests. B

...tient's serum has its own inherent gamma globulin and the action of the gamma globulin was regarded as the cause of the agglutination of plastic particles even if no sensitizing extra gamma globulin was added (*Singer* & *Plotz* 24, *Rheins, McCoy* & *Wall* 19. *Winblad* 30).

[1] Supported by grants from *Alfred Österlunds Stiftelse*.
I have to thank Mrs. *Britt Beronius* for technical assistance.

Another technical factor important for the agglutination reaction with plastic particles is the reaction temperature and the prozone phenomenon seen in association with the use of non-inactivated rheumatic serum. Such prozones have been interpreted as a manifestation of *inhibitors of thermolabile type* (*Schubart* 21, 22, *Brine, Wedgewood & Clark* 2).

In the discussion of the nature of the RAS factor it must be of importance to distinguish the different serum fractions in the reactant, which *are generally believed to be* gamma globulin, as well as in the reactor, *i.e.* that macroglobulin in rheumatic serum, which has been called the RAS factor. This is likewise of importance in the analysis of the nature of different inhibitors. Non-biological particles of latex type *or acryl can therefore be regarded* as particularly suitable for such studies.

On closer study of the agglutination of acryl particles in rheumatic serum it was observed that when these particles were suspended in *physiological saline or borate buffer, the* suspension was *labile in that* it agglutinated spontaneously when incubated at 56° C for 18 hours. This paper is concerned with an investigation of the effect of different serum fractions and different sera on acryl suspensions. That albumin *plays an important role in the stability of the* suspension was seem primary. Previously *Rhein, McCoy, Buehler & Burrel* (18) and *Rhein McCoy & Wall* (19) stressed that albumin can diminish the effect of the gamma globulin as an "antigen" in the reaction with the RAS factor.

METHODS

Acryl powder (polymethyl metacryl) with a particle size of 0.5 μ was supplied by AB Bofors. *1 g was suspended in 10 ml of distilled water and* shaken with glass beads and allowed to stand 20 minutes at room temperature for sedimentation. 0.5 ml of the supernatant was pipetted off and mixed with 0.9 ml borate buffer. The suspension obtained is of suitable even density and was used as stock solution. For a test 10 tubes were used with 0.1 ml of stock suspension of acryl + borate buffer up to 1.0 ml.

Borate buffer: 50 ml of 0.1 M boric acid, 5.9 ml of 0.1 M NaOH, distilled water to 100 ml (pH adjusted to 8.2) and 0.85 ml NaCl for each 100 ml.

Gamma globulin: Fraction II for therapeutic use from AB Kabi, Stockholm in 12 per cent dilution. Pure gamma globulin from the same firm was diluted with borate buffer.

Albumin from AB Kabi, Stockholm in 20 per cent dilution for therapeutic use.

Serum: Serologically positive rheumatoid arthritis serum and normal serum without demonstrable RAS factor were used after inactivation at 56° C for 30 minutes.

Acryl particle test: The serum was diluted twofold from a dilution 1:10 in the row of 15 tubes and to these tubes was added an equal amount of acryl suspension with *or* without sensitizing gamma globulin, albumin or sensitizing serum. Sensitizing serum and serum fraction, respectively, in the acryl suspension were used in the proportion 0.5 ml to 0.1 ml acryl suspension and borate buffer to 1.0 ml. Unless otherwise stated gamma globulin was present in a concentration of 1.2 per cent and albumin in 5 per cent solution. The reaction is allowed to proceed for 18 hours in a waterbath at 56° C and the results were read without centrifugation. Agglutination with *sedimentation* was said to be positive, an even suspension negative.

RESULTS

Reaction Temperature of Acryl Particle Test

The results of the acryl test conducted with acryl particles sensitized with gamma globulin against rheumatoid arthritic serum at different reaction temperatures for 18 hours are given in Table 1 No distinct agglutination *occurred at 4°, but this was distinct at 20° C, 37° C, and* 56° C with the highest titer at 56° C As expected, at 70° C, at which most of the proteins were denaturated, no agglutination occurred It was of interest to note that at 63° C, when the gamma globulin is aggregated and the best result is shown in the precipitation test, the agglutination is strongly diminished

TABLE 1

Acryl Particles Agglutination Titres by Different Reaction Temperature

	4°	20°	37°	56°	63°	70° C
Rheuma arth serum	?	1 280	2 560	2 560	160	0
Serum from healthy people	0	0	0	0	0	0

Labile Acryl Particle Suspension in Presence of Different Sera and Serum Fractions

In the absence of serum and at a temperature of 56° C the suspension of acryl particles used for the test agglutinated after 2 hours and to an equal extent after 18 hours Addition of such suspension without sensitizing serum or serum fraction to a dilution series of different sera or serum fractions gave the results summarized in Table 2 Spontaneous agglutination in the labile system ceased in the presence of albumin or in normal serum The stabilizing function is effective in such a high dilution as 1/5000 but not in dilution 1/10,000 or higher *The stabilizing function must be ascribed to the known effect of albumin as a protective colloid* (cf effect of albumin on cephalin cholesterol emulsion) (*Hanger* 13) The stabilizing effect of normal serum must be ascribed to the albumin which tolerates heating to 60° C and 63° C for 30 minutes and is thus thermostabile

Gamma globulin used as fraction II for therapeutic use (Kabi) showed no stabilizing effect, since agglutination occurred No inherent capacity of spontaneous agglutination was revealed because of the ab scence of a stabilizing back-ground But if this gamma globulin is heated at 60° C for 30 minutes a stabilizing function occurs up to a titer of 1/300 and after heating at 63° C for 30 minutes, to 1/600 The gamma globulin has then been converted into partly aggregated (60° C) or completely aggregated form (63° C) *The now demonstrable stabilization* of the labile system can be ascribed either to the occurrence in

TABLI 2

Acryl Particles Agglutination in Different Diluted Serums and Serum Fractions

Serum or serum fractions	Tube no												
	1	2	3	4	5	6	7	8	9	10	11	12	13
	20	40	80	160	320	[illegible]	1 280	2 560	5 120	10 240	20 480	[illegible]	[illegible]
Albumin (Kabi) 5 p c	—	-	—	—	—	—	—	—	—	++	++	++	++
60° 30 min	—		—	—		—	—	—	—	++	++	++	++
63° 30 min		—	—	—	—	—	—	—	—	++	++	++	+
Serum from healthy people	—		—	—	—	—	—	—	—	+	+	+	++
60° 30 min	-	—	—	—	—	—	-		(+)	+	+	+	++
63° 30 min		-	—	—	—	—	—	—	+	++	++	++	++
Gamma globulin (Kabi) 1 2 p c	++	++	++	++	++	++	++	++	++	++	++	++	++
60° 30 min	—	—	—	—	—	+	++	++	++	++	++	++	++
63° 30 min	—		—	—	—	—	+	++	++	++	++	++	++
Gamma globulin (Kabi 1 2 p c (mostly albuminfree)	++	++	++	++	++	++	++	++	++	++	++	++	++
60° 30 min	—	—	—	+	++	++	++	++	++	++	++	++	++
Rheumatoid arthrit serum	++	++	++	++	++	++	++	++	++	++	++	++	++
60° 30 min	+	+	+	+	+	+	(+)	—	++	++	++	++	++
63° 30 min	(+)	(+)	—	—	—		—	—	++	++	++	++	++
Rheumatic arthrit serum (Weak titre)	++	++	++	+	+	+	—	—	—	++	++	++	++
60° 30 min	++	++	++	+	+	—	—	—	-	++	++	++	++
63° 30 min	—	—	—	—	—		—	—	(—)	++	++	++	++
Transferrin (Kabi)	—	—	—	-	—	—	—	—	(—)	(+)	++	++	++
60° 30 min	—	—	—	—	—	—	—	(+)	+	+	++	++	++
63° 30 min	—	—	—	—	—	—	(-)	+	++	++	++	++	++

\+ agglutination — no agglutination

the preparation of albumin as a contaminant or to a property of the gamma globulin which is manifest only in aggregated gamma globulin This latter possibility appears less likely The occurrence of contaminating albumin in this gamma globulin for therapeutic use is well known by the manufacturers A gamma globulin poorer in albumin was also tried (Table 2) and after heating showed a slightly smaller inhibiting prozone The stabilizing effect of the albumin is, however, suppressed by unheated gamma globulin in this more concentrated form, which occurs in the preparation of fraction II and therefore this *gamma globulin should possess a certain inherent capacity to agglutinate*

The discovery of this, which is sensitive to heat, was thus made possible only by the observation of the albumin effect which becomes manifest in association with this heating process

Rheumatoid arthritis serum (serologically active) showed a strong agglutination of the acryl system If the titer is high, the agglutination will occur even at extremely high dilutions, which is probably due to spontaneous agglutination as soon as the diluted RAS factor ceases to be effective If the titer of the rheumatic serum is not quite so high, there will first appear an agglutination zone (Table 2) followed by a non-agglutinated zone with a stabilized system, which on further dilution is ag[illegible]

the first t

capacity c

tinated t

of the albumin which has a higher titer in such serum (1/10,000) than the RAS factor The agglutination in the last tubes may be regarded as spontaneous agglutination of the labile system *Rheumatic serum thus*

the stabilizing effect

the RAS factor in the

the titer of the rheu-

.he albumin need not become manifest, rheumatic agglutination being followed immediately by spontaneous agglutination In the labile system, then, albumin is a valuable stabilizing factor for exact measurement of high titers of agglutination of rheumatic sera

Like gamma globulin, if rheumatic serum is heated at 63° C [illegible]

less but the titer is only

thus differs from gamm

abolish the agglutinating effect of rheumatic serum It is possible that rheumatic serum heated in this way can exert its agglutinating effect even in the abscence of gamma globulin normally occurring in the serum

TABLI

Agglutination in Acryl Particle System in Presence o

Suspension Acryl 0 1 ml Borat buffert 9 8 ml Each tube 1 0 ml	Added to suspension	Concentration of albumin	Diluted serum fraction
	–		Albumin 1 p c
			Gamma glob 1 2 p c
	Alb 0 1 ml 0 05 p c	0 0005 p c	Gamma glob 1 2 p c
	Alb 0 1 ml 0 1 p c	0 001 p c	Gamma glob 1 2 p c
	Alb 0 1 ml 0 3 p c	0 003 p c	Gamma glob 1 2 p c
	Alb 0 1 ml 0 5 p c	0 005 p c	Gamma glob 1 2 p c
	Alb 0 1 ml 0 6 p c	0 006 p c	Gamma glob 1 2 p c
	Alb 0 1 ml 1 0 p c	0 01 p c	Gamma glob 1 2 p c
	Alb 0 1 ml 2 0 p c	0 02 p c	Gamma glob 1 2 p c

\+ agglutinati o

Relation between Albumin and Gamma Globulin in Instable System of Acryl Particles

It is clear from the experiments described above that albumin stabilizes the labile system of acryl particles, but minute amounts of albumin occurring, for example, in therapeutic gamma globulin (Kabi) cannot stabilize the system in the presence of gamma globulin, but it can do so after a major part or all of the gamma globulin has been aggregated by heating at 60° 63° C for 30 minutes This must imply that the effect of a minute amount of albumin is suppressed by gamma globulin In normal serum this gamma globulin does not appear to be present in a concentration high enough to suppress the stabilizing effect of the albumin The quantitative ratio between gamma globulin and albumin was studied in the acryl system in relatively different amounts, and the results are given in Table 3 (It should be observed that the minute amounts of contaminating albumin in therapeutic gamma globulin were ignored) The albumin showed a stabilizing effect on the acryl system even in a dilution 0 0015 per cent in the absence of gamma globulin The concentration of albumin necessary to counteract agglutination of the system in the presence of 1 2 per cent gamma globulin solution was found to be about 0 02 per cent On lowering of this concentration down to 0 001 per cent agglutination occurred, which must be ascribed to the inherent agglutinating effect of gamma globulin

rent Amounts of Albumin and Gamma Globulin

Tube no														
	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Dilution														
	20	40	80	160	320	640	1 280	2 [illegible]	5 120	10 240	20 480	40 960	81 920	163 840
	—	—	—	—	—	—	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
+	+	+	+	+	+	+	+	+	+	(+)	—	—	—	—
+)	(+)	(+)	(+)	(+)	—	—	-	—		—	—	—	—	—
+)	(+)	—	—	—	—	—	—	—	—	—	—	—	—	—
+)	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

n agglutination

The experiment also shows that the gamma globulin *per se* has an agglutinating effect on the acryl system, which remains concealed until the creation of a stabilizing background provided by the albumin The ratio between the quantities also explains why a normal amount of gamma globulin in serum in the presence of a normal amount of albumin *in the same serum cannot be demonstrated in the form of a separate agglutination*

Adhesion of Serum Fractions to Surface of Acryl Particles

Since particles used for the fixation test were suspended in *inter alia* diluted serum fraction (gamma globulin) it was considered of interest

Method Serum (rheumatoid arthritis serum and normal serum) in dilution 1/10 and gamma globulin (therapeutic Kabi) and albumin (Kabi) were mixed separately with a suspension of 0 1 ml of acryl and 9 9 ml [illegible] l portions were then particles 15 minutes for their

The results are given in Table 4 *The stabilizing effect of pure albumin and normal serum is evidently retained on the surface on the par-*

ticles However, the gamma globulin coated particles agglutinated In order to ascertain whether this agglutination was spontaneous or an effect of persistent gamma globulin, an experiment was performed (Table 5) in which gamma globulin coated and washed particles were tested against heated therapeutic gamma globulin containing minute amounts of albumin It was found that the minute amount of albumin otherwise capable of stabilizing the system of *uncoated particles*, cannot do so if the particles are coated with gamma globulin, which suggests that the gamma globulin attached itself to the surface of the particles and thereby give them the power of agglutination which cannot be stabilized by a small amount of albumin Partly aggregated gamma globulin (60° for 30 minutes) which had proved to lose its own power of agglutination, should therefore have a stabilized system because the minute amount of albumin attaches itself to the particles This was, however, not manifest, but suggests that the small amount of albumin had probably been washed off Agglutination of the system with particles coated with partly aggregated gamma globulin should therefore be ascribed to a spontaneous agglutination of the system

TABLE 4

Agglutination of Acryl Particles after Sensitization Centrifugation and Washing

Sensitized serum or serum fraction	Agglutination of acryl system	
	Without centrifugation or washing	After centrifugation and washing twice
Albumin	—	
Normal serum		—
Gamma globulin	+	+
Gamma globulin 60° 30 min		+
Rheumaserum	+	
Rheumaserum 60° 30 min	+	
Rheumaserum 63° 30 min	—	—

\+ agglutination — no agglutination

Rheumatic serum does not cause any agglutination of the particles after washing, despite the strong power of agglutination in the presence of rheumatic serum *The relatively large rheumatic factor molecules can thus not adhere to the particles in the same way as gamma globulin and albumin* This observation is agreement with the finding of *Coke* (5) regarding latex particles The stabilizing effect of the particle suspension is an effect of the serum albumin

Relation between Acryl System and an Intermediate Fraction (Transferrin)

Since it is thus clear that the gamma globulin causes agglutination while the albumin causes a stabilization of the acryl system it was considered of interest also to study how an intermediate fraction might

TABLE 5

Agglutination of Acryl System of Gamma Globulin after Sensitization with Gamma Globulin

Sensitizing serum fraction	Diluted serum fraction	Tube no										
		1	2	3	4	5	6	7	8	9	10	12
		Dilution										
		20	40	80	160	320	640	1 280	2 560	5 120	10 240	20 480
—	Gamma glob	+	+	+	+	+	+	+	+	+	+	+
—	Gamma glob 60° 30 min	—			—	(+)	+	+	+	+	+	+
Gamma glob	Gamma glob	+	+	+	+	+	+	+	+	+	+	+
Gamma glob	Gamma glob 60° 30 min	(+)	(+)	(+)	+	+	+	+	+	+	+	+
Gamma glob 60° 30 min	Gamma glob	+	(+)	(+)	(+)	(+)	+	+	+	+	+	+

+ agglutination, — no agglutination

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Sensitized serum or serum fraction	Agglutination of acryl system	
	Without centrifugation or washing	After centrifugation and washing twice
Albumin	—	—
Normal serum		—
Gamma globulin	+	+
Gamma globulin 60° 30 min		+
Rheumaserum	+	—
Rheumaserum 60° 30 min	+	—
Rheumaserum 63° 30 min	—	—

+ agglutination, – no agglutination

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		Dilution										
		20	40	80	160	320	640	1 280	2 560	5 120	10 240	20 480
—	Gamma glob	+	+	+	+	+	+	+	+	+	+	+
—	Gamma glob 60° 30 min	—		—	—	(+)	+	+	+	+	+	+
Gamma glob	Gamma glob	+	+	+	+	+	+	+	+	+	+	+
Gamma glob	Gamma glob 60° 30 min	(+)	(+)	(+)	+	+	+	+	+	+	+	+
Gamma glob 60° 30 min	Gamma glob	+	(+)	(+)	(+)	(+)	+	+	+	+	+	+

+ agglutination, no agglutination

TAB

Agglutination of Acryl Particles by Rheumat

Sensitizing serum or fractions (reactant)	Factors in reactant	Factors in reactor (rheumatic serum)	Dilution 20	40	80	160	320	640
None	None	RgA	++	++	++	++	++	–
Gammaglob (Kabi)	Ga	RgA	++	++	++	++	++	+
" 60° C 30 min	a	RgA	++	++	++	+	+	(–
" sensibil part *	G	RgA	++	++	++	++	++	+
" 60° C 30 min sensibil part *	None	RgA	++	++	++	++	++	(+
Albumin (Kabi)	A	RgA	++	++	++	+	+	+
Normal serum	gA	RgA	++	++	++	+	(+)	–
Rheumaserum	RgA	RgA	++	++	++	++	++	++
" 60° C 30 min	RA	RgA	++	++	++	++	++	++
sensibil part *	gA	RgA	++	++	++	++	++	++
" 60° C 30 min sensibil part *	A	RgA	++	++	++	++	++	+-

* washed, + agglutination

behave in this respect and for this purpose transferrin (with iron) was chosen that had been prepared in a form completely free (Kabi) from gamma globulin and from albumin It is clear from Table 2 that this also stabilized the system and that its stabilizing effect is thermostabile It thus appears that substances other than the serum albumin help to stabilize the plast particle system, which is labile, even though the stability, for simplicity, is ascribed below mainly to the albumin

Factors of Significance in the Agglutination of Acryl Particles

In the light of the above observations it may be justified to distinguish the following factors in the agglutination of acryl particles in a working hypothesis

Gamma globulin (fractionated, concentrated)	G
Gamma globulin in serum	g
Albumin fractionated or in serum	A
Albumin in min contaminating amounts in gammaglobulin	a
Rheumatoid arthritis factor	R

In their relation to the acryl system it may be stated that

(1) The stabilizing effect of albumin (A) is greater than the agglutinating effect of G

rum with Different Sensitizing Sera or Fractions

Dilution							Factors		
210	2 480	5 120	10 240	20 480	40 960	81 920	Tube no 1 5	6-10	> 10
–	–	+	++	++	++	++	RgA	(r)A	none (spont agglut)
+	++	++	++	++	++	++	*RGgAa*	*(r)GAa*	*Ga*
)	–	–	–	–	–	–	RgAa	(r)Aa	a
+)	+	++	++	++	++	++	RgGA	(r)AG	g
+)	(+)	+	++	++	++	++	RgA	(r)A	none (spont. agglut)
–	–	–	–	–	–	–	RgAA	(r)AA	A
–	–		–	–	–	–	RggAA	(r)gAA	gA
++	++	++	++	++	++	++	*RRggAA*	R(r)gAA	RgA
++	++	++	++	++	++	++	RRgAA	R(r)AA	RA
	–			–	–	–	RggAA	(r)gAA	gA
		–	–	–	–	–	RgAA	(r)AA	*A*

no agglutination

(2) The agglutinating effect of G exceeds the stabilizing effect of the minute amount of albumin (a)
(3) The agglutinating effect of the rheumatoid arthritis factor (R) exceeds the stabilizing effect of albumin (A)

In the description of different serum fractions the following abbreviations can be used and the following effects may be assumed

Gamma globulin (therapeutic Kabi)	Ga
Heated 60 63°	a
Sensitized particles, washed	G
Sensitizing particles with heated gammaglobulin washed no factor	
Albumin (Kabi)	A
Normal serum	gA
Normal serum heated at 60 63°	A
Serologically positive rheumative serum	RgA
heated 60° C 30 m	RA
– heated 63° C 30 m	A
sensitized particles with rheumatic serum, washed	gA
– sensitized particles with heated rheumatic serum	A

Behaviour of Sensitized Acryl Particles in Rheumatoid Arthritis Serum

The Latex fixation test, like the acryl fixation test used by us previously, is based on the assumption that the particles are sensitized by gamma globulin reacting in the form of *agglutination in rheumatic* serum One theory is that the gamma globulin reacts with the rheumatoid arthritis factor (fixation[!]) and that clumping of the particles is a result of this contact just as the precipitation is an expression of contact between the aggregated gamma globulin and the rheumatoid arthritis factor and the formation of a loose complex in gel form If, however, various sensitizing substances be analysed above such as those described and the agglutination be studied, the problem can be elucidated from further interesting angles The sensitizing factor may be regarded as a reactant "antigen" in the particle suspension or if the particles have been centrifuged off and washed, as a sensitizing factor coating the surface of the particles and reacting in the diluted serum studied which acts as a reactor Variation of different sensitizing serum or fractions as reactants against rheumatic serum in the dilution will give results shown in Table 6 In the experiments given in that table agglutination of the RAS factor occurred in the lowest dilutions in all forms of sensitization as well as in the absence of sensitizing reactant When the reactant contains RAS factor or gamma factor (G) and the latter wasn't mixed with albumin (A), the agglutination was shown in all tubes If the factors had been completely destroyed in the reactant as in sensitization with heated gamma globulin and washing with disappearance of even the minute amounts of albumin, the result would be teh same But if the reactant contain albumin in not too small amounts, the tubes in the other half of the dilution series will be stabilized and free from agglutination The intermediate tubes in the dilution series show much of interest A certain balance was observed between RAS factor described by dilution and the albumin present in the system Large amounts of albumin suppressed the small amounts of rheumatoid arthritis factor The RAS factor combined with gamma globulin could cause agglutination despite relatively large amounts of albumin present in the system Otherwise the presumed *balance between the different factors described above was clearly manifest in all of the combinations of dilutions* The experiments also showed that the *rheumatic arthritis factor can agglutinate even in the absence of unheated gamma globulin* This is apparent from experiment when rheumatic serum heated at 60° acts both as a reactant and a reactor

Acryl Particle Agglutination in Presence of Albumin

It is apparent from the observations set forth above that the acryl system must be stabilized to prevent spontaneous agglutination especially because of the fact that some rheumatic sera can give such strong

TABLE 7

Agglutination of Acryl Particles with Different Amounts of Sensitized Gamma Globulin and Albumin and by 0 1 ml Diluted Rheumatic Serum

Sensitized serum fraction added to 0 1 ml of acryl suspension and borate buffer to final volume of 2 0 ml		Tube nr												
		1	2	3	4	5	6	7	8	9	10	11	12	13
		Dilution												
Gamma globulin	Albumin	20	40	80	160	320	640	1 280	2 560	5 120	10 240	20 480	40 960	81 920
1 0 ml 5 p c (0 5 p c)	0 1 ml 5 p c (0 05 p c)	++	++	++	++	++	++	++	++	++	++	++	++	++
1 0 ml 2 5 p c (0 25 p c)	0 1 ml 5 p c (0 05 p c)	++	++	++	++	++	++	++	++	++	(+)	(+)	(+)	(+)
1 0 ml 1 2 p c (0 12 p c)	0 1 ml 5 p c (0 05 p c)	++	++	++	++	++	++	++	(+)	—	—	—	—	—
0 5 ml 1 2 p c (0 06 p c)	0 1 ml 5 p c (0 05 p c)	++	++	++	++	++	++	++	(+)	—	—	—	—	—
0 25 ml 1 2 p c (0 03 p c)	0 1 ml 5 p c (0 05 p c)	++	++	++	++	++	++	+	(—)	—	—	—	—	—
0 25 ml 1 2 p c (0 03 p c)	0 1 ml 1 p c (0 01 p c)	++	++	++	++	++	++	++	++	+	(+)	(+)	(+)	(+)
0 25 ml 1 2 p c (0 03 p c)	0 1 ml 0 5 p c (0 005 p c)	++	++	++	++	++	++	++	++	++	++	++	++	++

\+ agglutination — no agglutination

Behaviour of Sensitized Acryl Particles in Rheumatoid Arthritis Serum

The Latex fixation test, like the acryl fixation test used by us previously, is based on the assumption that the particles are sensitized by gamma globulin reacting in the form of agglutination in rheumatic serum One theory is that the gamma globulin reacts with the rheumatoid arthritis factor (fixation[1]) and that clumping of the particles is a result of this contact just as the precipitation is an expression of contact between the aggregated gamma globulin and the rheumatoid arthritis factor and the formation of a loose complex *in gel form* *If, however,* various sensitizing substances be analysed above such as those described and the agglutination be studied, the problem can be elucidated from further interesting angles The sensitizing factor may be regarded as a reactant "antigen" in the particle suspension or if the particles have been centrifuged off and washed, as a sensitizing factor coating the surface of the particles and reacting in the diluted serum studied, which acts as a reactor Variation of different sensitizing serum or fractions as reactants against rheumatic serum in the dilution will give results shown in Table 6 In the experiments given in that table agglutination of the RAS factor occurred in the lowest dilutions in all forms of sensitization as well as in the absence of sensitizing reactant When the reactant containes RAS factor or gamma factor (G) and the latter wasn't mixed with albumin (A), the agglutination was shown in all tubes If the factors had been completely destroyed in the reactant, as in sensitization with heated gamma globulin and washing with disappearance of even the minute amounts of albumin, the result would be teh same But if the reactant contain albumin in not too small amounts, the tubes in the other half of the dilution series will be stabilized and free from agglutination The intermediate tubes in the dilution series show much of interest A certain balance was observed between RAS factor described by dilution and the albumin present in the system Large amounts of albumin suppressed the small amounts of rheumatoid arthritis factor The RAS factor combined with gamma globulin could cause agglutination despite relatively large amounts of albumin present in the system Otherwise the presumed *balance between the different factors described above was clearly manifest in all of the combinations of dilutions* The experiments also showed that the *rheumatic arthritis factor can agglutinate even in the absence of unheated gamma globulin* This is apparent from experiment when rheumatic serum heated at 60° acts both as a reactant and a reactor

Acryl Particle Agglutination in Presence of Albumin

It is apparent from the observations set forth above that the acryl system must be stabilized *to prevent* spontaneous agglutination especially because of the fact that some rheumatic sera can give such strong

agglutination of acryl particles sensitized with gamma globulin that the RAS factor in dilution series is followed directly by spontaneous agglutination of the system The amount of albumin must be adjusted according to the gamma globulin in the acryl suspension, so that the albumin affect is not too low and the stabilizing effect thereby suppressed Table 7 gives a comparison between different concentrations of gamma globulin and albumin in the acryl suspension in a test of a serologically strongly active rheumatoid arthritis serum It is obvious from the table that the concentration of the albumin in the suspension should be more than 0 05 per cent and the amount of globulin should not be more than 2½ times as large

A suitable addition to the acryl suspension (0 1 mm acryl suspension + borate buffer to a final volume of 10 ml) is 0 5 ml of 1 2 per cent gamma globulin and 0 5 ml of 5 per cent albumin Table 8 shows how rheumatic serum by such addition of gamma globulin in sufficient amount of albumin can be measured in titer values which are much higher than what can be reached if the rheumatic serum is titrated in an acryl suspension with albumin alone and with the gamma globulin inherent in the serum That the presence of the extra gamma globulin is the cause of the higher titers is apparent from the reduction of the titer on heating of the added gamma globulin as well as heating of the gamma globulin in the rheumatic serum The concentrations of gamma globulin recommended above and of the albumin, have proved advantageous in practice partly because the amounts of these serum fractions added can be kept constant and partly because weak agglutinating effects in rheumatic serum can achieve a measurable value

As mentioned previously, Table 8 shows that the gamma globulin appears if anything to give an *additive effect rather than to be a manifestation of a necessary fixation between itself and the rheumatic factor*

DISCUSSION

The present investigation elucidated the necessity of stabilization of the plast particle agglutination and stressed the role of albumin as a stabilizing factor (protective colloid) *Rhein* et al (18, 19) also emphasized the role a shift in the balance between the gamma globulin and albumin can influence the result It must therefore be of importance that these two substances are well balanced and constantly of the same concentration

[illegible] of plast particles This is perhaps not surprising in the light of the observations by *Franklin et al* (7 8) that the rheumatic factor is large (19 S or 22 S) compared with that of the gamma globulin (7 S) *Lospaluto & Ziff*

TABLF 8

Acryle Particles Agglutination of Rheuma Serum in Different Sensitizing Milieu

Sensitizing serum fraction to 0 1 ml acryl suspension and borate buffert to 10 ml	Diluted serum	Tube no 1	2	3	4	5	6	7	8	9	10	11	12	13
		Dilution 20	40	80	160	320	640	1 210	2 480	4 560	9 120	18 280	32 560	65 120
None	Rheuma serum	++	++	++	++	++	++	+	—	—	++	++	++	++
Albumin 0 5 ml 5 p c		++	++	++	++	+	+	—	—	—	—	—	—	—
Gammaglob 0 5 ml 1 2 p c		++	++	++	++	++	++	++	++	++	++	++	++	++
Albumin 0 5 ml 5 p c + Gammaglob 0 5 ml 1 2 p c		++	++	++	++	++	++	++	++	++	(+)	(—)	—	—
Albumin 0 5 ml 5 p c + Gammaglob 0 5 ml 1 2 p c 60° C 30 min		++	++	++	++	+	+	—	—	—	—	—	—	—
Albumin 0 5 ml 5 p c + Gammaglob 0 5 ml 1 2 p c 60° C 30 min	(60° C 30 min)	++	++	++	+	—	—	—	—	—	—	—	—	—

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		Dilution												
		20	40	80	160	320	640	1 210	2 480	[illegible]	9 120	18 280	32 [illegible]	[illegible] 120
None	Rheuma serum	++	++	++	++	++	++	+	—	—	++	++	++	++
Albumin 0 5 ml 5 p c		++	++	++	++	+	+	—	—	—	—	—	—	—
Gammaglob 0 5 ml 1 2 p c		++	++	++	++	++	++	++	++	++	++	++	++	++
Albumin 0 5 ml 5 p c Gammaglob 0 5 ml 1 2 p c		++	++	++	++	++	++	++	++	++	(+)	(—)	—	—
Albumin 0 5 ml 5 p c Gammaglob 0 5 ml 1 2 p c 60° C 30 min		++	++	++	++	+	+	—	—	—	—	—	—	—
Albumin 0 5 ml 5 p c Gammaglob 0 5 ml 1 2 p c 60° C 30 min	(60° C 30 min)	++	++	++	+	—	—	—	—	—	—	—	—	—

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By means of a suitable balance between the albumin and gamma globulin in the suspension of plast particles the agglutination reaction can be modified so that the titer of serologically active serum can be measured in higher values A practical application of this reaction is described

Judging from the investigations the inherent agglutinating capacity of rheumatic sera probably does not require the presence of gamma globulin although the presence of the latter increases the strength of the reaction considerably It is open to discussion whether the term fixation test is theoretically correct The possibility of an additive agglutinating function of rheumatic serum and gamma globulin seems likely

REFERENCES

1 *Bloch K J & Bunim J J* Simple rapid diagnostic test for rheumatoid arthritis Bentonite flocculation test J A M A 169 307 1959
2 *Brine K L Wedgewood R J & Clark W S* The effect of serum complement and its components on the rheumatoid latex fixation test Arth Rheumat *1* 230 1958
3 *Burby G & Behr G* The latex fixation test in rheumatoid arthritis Lancet 2 1157 1958
4 *Christian Ch L* Properties of aggregated human gamma globulin Arthrit Rheumat 3 268 1960
5 *Coke H* The nature of the rheumatoid macro globulin Protides of the biologic fluids Elsevier Amsterdam 376 1960
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7 [illegible]
8 [illegible]
J Immun 85 138 1960
9 *Franklin E C* Stabilizers and inhibitors of the sensitized sheep cells agglutina [illegible]
10 *Gofto* [illegible]
11 *Crub* [illegible]
[illegible] Basel
12 *Grubb R* Interactions between Rheumatoid Arthritis Sera and Human Gamma Globulin Acta [illegible]
13 *Hanger F M* The [illegible] by patho logical sera T [illegible]
14 *Lospalluto J & Zi* [illegible] toid factor J Exp Med *1* [illegible]
15 *Manik M Brine K L & Clark W S* Observations concerning the reactivity of normal and rheumatoid gamma globulin in rheumatoid serological reactions Arth & Rheumat *1* 460 1958
16 Müller W Eine einfache *Pracipitationsreaktion* zum qualitativen Nachweis des Rheumafaktors" Klin Wochenschr 37 86 1959
17 *Lit A A* De mastix fixation *reactie bij* reumatoid arthrit Ned T Geneesk *103* 2310 1959

(14), who *purified different* constituents of fraction II of a rheumatic serum, found that haemagglutination is linked only to one of these fractions and therefore suggested the possibility that the different serological *manifestations* of the rheumatic factor might be due to different parts of a complex system which, however, are uniform in the sedimentation constant

The *purpose* of the *present investigation with an albumin-stabilized* acryl system was to distinguish the influences of the different serum fractions on the system and thereby hypothetically to distinguish factors of significance in agglutination It *appears that the rheumatic* factor can cause agglutination even in the absence of unheated gamma globulin The name fixation test may appear inadequate and can perhaps be replaced by "addition test" The function of the rheumatic factor would then be conceived as an agglutination activating factor or perhaps an entirely independent agglutinating factor

These experiments were carried out with acryl particles It was not possible for us to supplement them with similar experiments with Latex particles It might be assumed that relatively similar laws hold for other plast particles as for acryl particles

SUMMARY

After 1 day's incubation at 56° C acryl plast particles in borate buffer showed spontaneous agglutination This can be stabilized with albumin or transferrin as well as with normal serum in which the albumin is probably the most active component Fractionated gamma *globulin* and serum from patients with serologically active rheumatoid arthritis agglutinates the particles, which is distinct in a suspension stabilized *with albumin The inherent capacity of agglutination of* the gamma globulins, however, becomes manifest only in the presence of minute amounts of albumin and is suppressed by larger amounts of albumin The agglutination capacity of rheumatic serum and gamma globulin are thermolabil and inactivated by heating at 63° C for 30 minutes After 30 minutes at 60° C the agglutinating effect of the gamma globulin (Fraction II Kabi) is inactivated, while the effect of rheumatic serum is only slightly reduced The agglutination of rheumatic serum is largely independent of the amount of albumin

The rheumatic factor has no tendency to adhere to the surface of acryl particles and disappears after centrifugation and washing of *the* particles If the amount of albumin is not too small, it will adhere to the surface of the acryl particles and have a stabilizing effect on the suspension *Gamma globulin, identified by its agglutinating effect,* adheres to the surface of the particles

The effect of albumin, gamma globulin and rheumatoid arthritis factor was studied in such an acryl system and it was found that the rheumatoid arthritis factor showed an agglutinating effect even when

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[illegible] 1959 Basel

12 *Grubb R* Interactions between Rheumatoid Arthritis Sera and Human Gamma Globulin Acta Haematologica *20* 246 1958

13 *Hanger F M* The flocculation of encephalin cholesterol emulsions by pathological sera Tr Ass Am Physicians 53 148 1958

14 *Lospalluto J & Ziff M* Chromatographic studies of the rheumatoid factor J Exp Med *110* 169 1959

15 *Manik M Brine K L & Clark W S* Observations concerning the reactivity of normal and rheumatoid gamma globulin in rheumatoid serological reactions Arth & Rheumat *1* 460 1958

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STUDIES ON THE INACTIVATION OF BACTERIAL VIRUSES BY NORMAL HUMAN SERUM[1]

5 *The Influence of Heated and Zymosan Adsorbed Serum on the Inactivation of Coli T2 Phages*[2]

By

LARS OLOF KALLINGS[3]

Received 25 ii 61

Depending on the dose, heated or zymosan adsorbed serum may exhibit an inhibiting or enhancing effect on the neutralization by normal serum of coli T2r+ phages This effect was revealed when heated or zymosan treated homologous sera were used as diluents in attempts to straighten the sigmoid inactivation—serum concentration curve by making the reaction mixtures homogeneous with respect to the content of regular serum components (*Kallings* 1958) In the investigation to be reported two principal types of experiments were performed to study this phenomenon (1) Constant or varying doses of heated or zymosan adsorbed serum were added to mixtures of phage and serial dilutions of fresh serum The neutralization was determined after a fixed reaction time (2) The progress of neutralization was followed at certain intervals in a mixture of phage, fresh and heated serum

MATERIAL AND METHODS

For preparation of RP (according to *Pillemer et al* 1956) zymosan (Standard Brands Inc Lots No 7B 13) was boiled for one hour in saline centrifuged at 3 500 r p m for 30 minutes and resuspended in 0 15 M veronal buffer (*Kabat & Mayer* 1948) Three mg zymosan in 0 08 ml veronal buffer was added per ml fresh human serum The suspension was stirred at 17 ± 0 3° C for one hour and then centrifuged at 3 500 r p m in the cold for one hour The procedure was repeated once The resulting reagent was tested as described below

If serum or RP were heated they were allowed to cool prior to the addition to the mixtures of phage and fresh serum The other materials and methods were described in previous papers (*Kallings* 1961 a c) "Homologous" serum in the present paper means serum from the same donor

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...son is gratefully acknowledged

18 *Rheins M S McCoy F W Buehler E V & Burrell R G* Effects of animal sera and serum albumin on latex fixation test for rheumatoid arthritis Proceed Soc Exp Biol Med *16* 67 1957
19 *Rheins M McCoy F W & Wall R I* Reactivity of globulins from rheumatoid sera in latex fixation test Proceed Soc Exp Biol Med *97* 632 1958
20 *Rose H M Regan C Pearce E & Lipman M O* Differential agglutination of normal and sensitized sheep erythrocytes by sera of patients with rheumatoid arthritis Proc Soc Exp Biol Med *70* 475, 1949
21 *Schubart A F Cohen A S & Calkins F* Latex fixation test in rheumatoid arthritis 1 Clinical significance of a thermolabile inhibitor New Engl J M *261* 363 1959
22 *Schubart A F* Latex fixation test in rheumatoid arthritis 2 Characterization of the thermolabile inhibitor by serologic study New Engl J M *261* 579 1959
23 *Singer J M & Plotz Ch M* The latex fixation test Application to the serologic diagnosis of rheumatoid arthritis Americ J Med *21* 888 1956
24 *Singer J M & Plotz Ch M* The latex fixation test for rheumatoid arthritis using patient s own gamma globulin Arthrit Rheumat *1* 142 1958
25 *Waaler F* On the occurrence of a factor in human serum activating the specific agglutination of sheep blood corpuscles Acta path et microbiol scandinav *17* 172 1940
26 *Vaughan J H* Serum responses in rheumatoid arthritis Americ J Med *26* 596 1959
27 *Vaughan J H Ellis P Jo & Marshall H* Quantitative considerations of the rheumatoid factor J Immun *81* 261 1958
28 *Winblad S* Studies on different laboratory tests for the rheumatoid arthritis serum factor 1 Evaluation of different agglutination methods for whole serum cold precipitable and euglobulin serum fractions Acta path et microbiol scandinav *49* 499 1960
29 *Winblad S* Studies in the laboratory estimation of the rheumatoid arhtritis serum factor 2 Gamma globulin precipitation test in relation to haemagglutination test *with sensitized sheep cells and acrylplast fixation test* Acta path et microbiol scandinav *49* 515 1960
30 *Winblad S* Studies on different laboratory tests for the rheumatoid arthritis serum factor 3 Slide agglutination methods with and without sensitizing gamma globulin Acta path et microbiol scandinav *49* 523 1960
31 *Zaranal V* Agglutination der mit Gamma Globulin sensibilisierten Kollodiumteilchen bei der chronischen Polyarthritis Zeitschr Rheumaforsch *17* 41 1958

FROM THE NATIONAL BACTERIOLOGICAL LABORATORY, STOCKHOLM

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[1] Aided by grants from the [illegible]

EXPERIMENTAL

Influence of Heated Homologous Serum on the Reaction between Phage and Fresh Normal Serum

Effect on the phage inactivation—serum concentration curve A serial dilution of native serum in 1 5 fold steps was made up using homologous serum heated at 56° C for 30 minutes as diluent The serum dilutions were mixed with equal parts of phages diluted in barbiturate buffer For comparison, barbiturate buffer was used as diluent instead of heated serum in a second series of reaction mixtures All mixtures were incubated at 37° C for one hour and treated according to the standard inactivation procedure described previously (*Kallings* 1961 a) As seen in Fig 1 the presence of heated serum in the reaction mixture remarkably influenced the inactivation rate The neutralizing activity in the low concentrations of native serum (*i e* high concentrations of heated serum) was completely abolished whereas it remained unchanged in the highest concentration of native serum tested

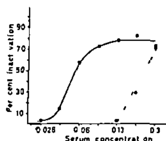


Fig 1

The inhibiting effect of heated serum on the phage inactivating capacity of various dilutions of fresh serum Abscissa Arithmetical values of the fresh serum concentrations on a log scale Left curve Fresh serum diluted with barbiturate buffer Right curve Fresh serum diluted with homologous serum heated at 56° C for 30 minutes —Reaction mixture 0 5 ml serum (1 1) dilution and about 1×10^4 infectious T2 particles diluted in 0 5 ml barbiturate buffer 0 125 M pH 7 4 ± 0 05 0 0025 M Mg^{++} Phage controls 0 5 ml nutrient broth + 0 5 ml phage dilution *Incubated at 37° C for 1 hour Duplicate experiment*

In the following series of experiments *constant* volumes of homologous sera heated at 56° C for 30 minutes were added to the reaction mixtures of phage and serial dilutions of native sera in barbiturate buffer The resulting mixtures were incubated at 37° C for 2 hours The dose of heated sera added was varied from series to series Four different sera were tested with consistent results Table 1 lists the percentages of inactivated phages at different concentrations of native and heated serum Similar to the findings in the experiment illustrated in Fig 1, the inhibiting effect of heated serum was less marked at high concentrations of native serum though a constant dose of heated serum was added The inhibiting effect was found to decrease with the dose of heated serum At a concentration 1 50 of heated serum in the reaction

mixture the inactivation process was not inhibited Below this limit, however, the effect of the heated serum was reversed Low concentrations of heated serum were thus found to enhance the phage inactivating ability of native serum

TABLE 1

Influence of Various Concentrations of Heated Serum on the Inactivation of Phages by Normal Serum Per Cent Inactivated Phages at Different Dilutions of Native Serum

Final dil of native serum in the reaction mixture	Final dil of heated serum in the reaction mixture				
	1 5	1 10	1 50	1 100	—
1 3 3	77 6	84 4	78 6	90 2	85 1
1 5	72 1	79 0	82 7	85 5	82 8
1 7 5	43 6	70 1	76 8	83 7	75 5
1 11 2	40 0	57 2	71 4	81.2	77 9
1 16 9	9 1	28 2	70 8	75 6	70 8
1 25.3	0	11 5	30 4	42 7	32 2
1 38 0	0	3 0	9 5	8 5	2 2
Reciprocal of serum dil inactivating 50 per cent of the phages	7 2	12 5	20 8	23 8	21 0

Reaction mixture About 1 × 10⁴ T2 phages + serum (A L A) dil in barbiturate buffer pH 7 4 ± 0 05 0 125 M + serum (A L A) heated at 56° C for 30 min Mg⁺⁺ added to 0 0025 M Incubated for 2 hours at 37° C

TABLE 2

Influence of Heated Serum on the Phage Inactivating Capacity of Normal Serum

Final dil of heated serum in the reaction mixture	Reciprocal of dil of normal serum inactivating 50 per cent of the phages	Per cent of original inactivating capacity
	29 7	100 0
1 10	14 4	48 5
1 12 5	18 4	62 0
1 15	20 2	68 0
1 25	29 5	99.3
1 50	41 5	139 7
1 100	43 5	146 5
1 200	34 2	115 2
1 400	26 7	89 9

Reaction mixture About 1 × 10⁴ T2 phages + serum (A Ö) dil in 1.5 fold steps in barbiturate buffer pH 7 4 ± 0 05 0 125 M + serum (A Ö) heated at 56° C for 30 min Mg⁺ added to 0 0025 M Incubated for 2 hours at 37° C.

Table 2 shows the result of a similar experiment with another ...
In th ...

... phage inactivating ability of native serum

Optimum enhancement of the inactivation occurred when heated serum was present in dilution 1 : 100. At this dilution the original phage inactivating capacity was increased by 46.5 per cent. At a tenfold higher concentration the inactivating capacity was decreased by 51.5 per cent.

In the above experiments the hemolytic complement activity in the reaction mixtures was measured on sensitized sheep erythrocytes according to a procedure described previously (*Kallings* 1961 a). The addition of heated serum was not found to alter the original hemolytic activity of the fresh sera (120–250 50 per cent hemolytic units, total volume of the test system 1.25 ml).

Effect of heated serum on the time-survival curves. To facilitate the interpretation of the results reported above, the kinetics were studied by conventional time-survival experiments.

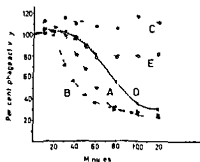


Fig. 2.
Influence of different concentrations of heated serum on the progress of the neutralization reaction.

Reaction mixtures

A. 0.04 ml normal serum + 0.46 ml buffer + 0.5 ml phage suspension + 0.2 ml buffer
B. 0.04 ml normal serum + 0.46 ml buffer + 0.5 ml phage suspension + 0.1 ml buffer + 0.1 ml heated serum
C. 0.04 ml normal serum + 0.46 ml buffer + 0.5 ml phage suspension + 0.2 ml heated serum
D. 0.025 ml normal serum + 0.475 ml buffer + 0.5 ml phage suspension + 0.2 ml buffer
E. 0.025 ml normal serum + 0.475 ml buffer + 0.5 ml phage suspension + 0.1 ml buffer + 0.1 ml heated serum

Phage activity at zero time determined with the aid of 4 broth controls (0.5 ml phage suspension containing about 1×10^4 infectious particles + 0.5 ml nutrient broth). Barbiturate buffer 0.125 M, pH 7.4 + 0.05 used as diluent. Mg^{++} added to 0.0025 M.

Two chilled mixtures of phage suspension and native serum (A A) were made up. The serum was finally diluted 1 : 25 (I) and 1 : 40 (II). The mixtures were divided into 1 ml aliquots and placed in a waterbath at 37° C after the addition to each aliquot of 0.2 ml diluted or undiluted homologous serum pre-heated at 56° C for 30 minutes or after the addition of 0.2 ml barbiturate buffer. The aliquots were kept cold until they were simultaneously placed in the waterbath. Duplicate mixtures were taken from the waterbath at the intervals shown in Fig. 2 and the reaction was stopped by chilling and dilution according to the standard

procedure (*Kallings* 1961 a) The curves A and D in the figure illustrate the progress of the phage inactivation with serum dilution I and II without any heated serum added The curves B and F show the effect of heated serum diluted 1 12 in the mixtures I and II respectively The same dilution of heated serum enhanced the phage neutralizing action of the higher concentration of native serum and inhibited the effect of the lower concentration When the concentration of heated serum was doubled the increase was converted to a complete inhibition for the re action periods observed (curve C as opposed to B) As shown in this same curve the number of infectious particles was on an average 12 per cent higher than revealed by the controls (equal volumes of phage suspension and nutrient broth) for determining the initial phage titer in the reaction mixtures This increase may be ascribed to the phage activating effect found in native as well as in heated normal sera (*Kal lings* 1961 a d)

The next experiment was designed to show the significance of small variations in the concentration of native serum on the effect of a con stant dose of heated serum Appropriate concentrations of fresh serum had previously been selected by preliminary experiments In the final experiment three chilled mixtures of phage suspension and native se rum (A L A) were made up having the final serum concentrations 1 30 0 (I) 1 31 7 (II) and 1 33 3 (III) As in the preceding experi ment the mixtures were divided into 1 ml aliquots and were kept cold To half the number of aliquots of each serum concentration 0 1 ml homologous serum heated at 56° C for 30 minutes was added and to the other half 0 1 ml barbiturate buffer The aliquots in duplicate were incubated at 37 C for the same time intervals as in the preceding ex periment The result is shown in Fig 3 The regular progress of inacti vation is represented by the data obtained from mixture II without heated serum (curve A) The inactivation in mixtures I and III without heated serum proceeded at about the same rate as II I somewhat faster and III somewhat slower Curves B C and D show the influence of heated serum on the mixtures I II and III respectively It is evident that the concentration of heated serum used markedly retarded the phage neutralizing action of the serum diluted 1 33 3 (III) though it only slightly influenced the action of the same serum diluted 1 30 (I)

In the following experiment the extended course of the curves A and D was followed to investigate if the phage neutralization was perma nently inhibited or only delayed This experiment was set up in a way analogous to the preceding one the inactivation reached after 100 180 minutes and after 20 hours being determined Twenty hours was chosen as the phage inactivation caused by normal serum was previously shown to continue up to this time (*Kallings* 1961 a) As shown in Fig 4 the inactivation proceeded slowly with time and uniformly in both mixtures At this ratio between native and heated serum the number of infectious phages thus remained on a high level even after prolonged

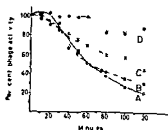


Fig 3
Influence of a constant concentration of heated serum on the progress of phage neutralization caused by different concentrations of normal serum

Reaction mixtures
A 0 0315 ml normal serum + 0 4685 ml buffer + 0 5 ml phage suspension + 0 1 ml buffer
B 0 033 ml normal serum + 0 467 ml buffer + 0 5 ml phage suspension + 0 1 ml heated serum
C 0 0315 ml normal serum + 0 4685 ml buffer + 0 5 ml phage suspension + 0 1 ml heated serum
D 0 030 ml normal serum + 0 470 ml buffer + 0 5 ml phage suspension + 0 1 ml heated serum
For further experimental details see text to Fig 2

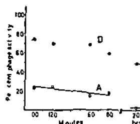


Fig 5
Influence of heated serum on the progress of the neutralization reaction (The extended course of curves A and D in Fig 3)
See text to Fig 3

incubation This observation was confirmed by an experiment in which a constant volume of serum heated at 56° C for 30 minutes was added to reaction mixtures of phage and serial dilutions of native serum (0 1 ml heated serum per ml phage-serum mixture) The mixtures were then incubated in a waterbath at 37° C for 20 hours The result is illustrated in Fig 5 A dilution of fresh serum 1 19 6 was necessary to inactivate 50 per cent of the phages when heated serum was present The same inactivation was brought about by the fresh serum diluted 1 110 when no heated serum was added

A comparison was made between the neutralization-inhibiting influences of serum heated at 50° and 56° C for one hour 0 1 ml heated serum was added to 1 ml aliquots of a mixture of phage and fresh serum (A L A) diluted 1 33 3 The progress of phage inactivation was followed by time-survival experiments conducted as those described above After exposure to 50° C the heated serum was found to inhibit the neutralizing process but to a lesser extent than after exposure to 56° C

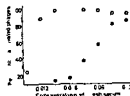


Fig 5

Inh b ting effect of a constant concentration of heated serum on phage neutraliza t on in serial dilutions of nat e serum furing 20 hours at 37° C
Open circles No heated serum added Filled circles Heated serum added

Abscissa Arithmetical values of serum concentration on a log scale
Reaction mixture 0 5 ml serum (A L A) dilution + 0 5 ml phage suspension (about 1×10^{4} T2 part cles) 0 1 ml buffer or heated serum added Phages and serum di luted in barbiturate buffer 0 125 M pH 7 4 ± 0 05 Mg 0 0025 M

Influence of Serum Adsorbed with Zymosan on the Reaction between Phage and Normal Serum

Serum (A A) was treated with zymosan with the intention of re moving the phage neutralizing ability without inactivating the com plement factors The resulting reagent (RP) was tested for hemolytic activity and for remaining phage inactivating capacity according to standard procedures (*Kallings* 1961 a) The reagent was found to con tain 137 50 per cent hemolytic units per ml No phage inactivating effect was demonstrable after two hours at 37° C nor at very high concentra tions of the reagent (1 1 5) No test was made to examine if the zymo san adsorbed serum was able to destroy C 3 at 37° C after addition of zymosan due to remaining properdin The designation RP may there fore be improper but is used for practical purposes

As mentioned in the introduction the inhibitory effect of RP was observed when the reagent was used in attempts to standardize the phage inactivation test The result of such an attempt is shown in Fig 6 Curve A represents the course of inactivation when only phage and various dilutions of native serum were mixed and incubated for two hours at 37° C Curves B and C show the course of inactivation when RP was added to all the dilutions of native serum to give a con stant total serum concentration (native serum + RP) of 1 5 and 1 10 respectively Some straightening of the inactivation curve was obtained and the curve was displaced towards higher concentrations of fresh serum when the higher concentrations of RP were added (curve B) A more marked flattening of the curve occurred when a constant dose of RP was added to the reaction mixtures (0 2 ml RP per ml) as indicated by curve D The effect of the same dose of RP heated at 56° C for 30 minutes was also tested The heated reagent exhibited a very strong inhibiting effect on the neutralization reaction (curve F) Heated RP was found to be more powerfully inhibiting than untreated serum heated at 56° C for 30 minutes (curve F 0 2 ml heated serum per ml reaction mixture)

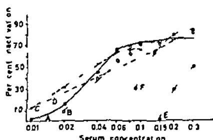


Fig 6
Influence of varying and constant concentrations of RP on the phage neutralization in various dilutions of native serum

Abscissa Arithmetical values of fresh serum concentrations on a log scale Reaction mixtures A 0 5 ml serum (I M) dilution + 0 5 ml phage suspension (about 1×10^4 T2 particles) 0 0025 M Mg^{++} Diluent Barbiturate buffer, 0 125 M pH 7 4 ± 0 05 0 2 ml buffer added to the reaction mixture Incubated at 37° C for 2 hours B similar to A but RP added to the decreasing amounts of native serum to a total volume of 0 2 ml C similar to B but RP added to a total volume of 0 1 ml D similar to A but 0 2 ml RP instead of buffer added to each ml of reaction mixture E similar to D but 0 2 ml RP heated at 56° C for 30 minutes prior to addition F similar to F but 0 2 ml heated serum added instead of RP Means of duplicate experiments

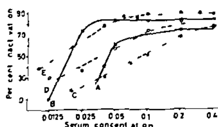


Fig 7
Influence of different constant concentrations of RP on phage neutralization in serial dilutions of native serum during 2 and 4 hours at 37° C

Abscissa Arithmetical values of fresh serum (A 0) concentrations on a log scale
Reaction mixtures
A 0 5 ml dil of normal serum + 0 5 ml phage suspension + 0 2 ml buffer Incubated for 2 hours
B 0 5 ml dil of normal serum + 0 5 ml phage suspension + 0 2 ml buffer Incubated for 4 hours
C 0 5 ml dil of normal serum + 0 5 ml phage suspension + 0 2 ml RP Incubated for 2 hours
D 0 5 ml dil of normal serum + 0 5 ml phage suspension + 0 2 ml RP Incubated for 4 hours
F 0 5 ml dil of normal serum + 0 5 ml phage suspension + 0 06 ml RP Incubated for 2 hours
For further experimental details see text to Fig 2

The influence of a constant dose of RP is further elucidated by experiments illustrated in Fig 7 A mixture of phage and a twofold serial dilution of native serum was made up and divided into 1 ml aliquots Duplicate aliquots were incubated for two (curve A) and four hours (curve B) at 37° C after the addition of *0 2 ml barbiturate buffer* RP, 0 2 ml per ml reaction mixture, was added to other duplicate aliquots

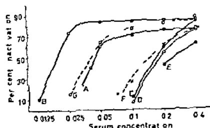


Fig 8

Influence of different constant concentrations of heated RP on phage neutralization in serial dilutions of native serum during various periods at 37° C

Abscissa Arithmetical values of fresh serum (A 0) concentrations on a log scale

Reaction mixtures

A 0 5 ml dil of normal serum + 0 5 ml phage suspension + 0 2 ml buffer Incubated for 2 hours
B 0 5 ml dil of normal serum + 0 5 ml phage suspension + 0 2 ml buffer Incubated for 4 hours
F 0 5 ml dil of normal serum + 0 5 ml phage suspension + 0 2 ml heated RP Incubated for 1 hour
D 0 5 ml dil of normal serum + 0 5 ml phage suspension + 0 2 ml heated RP Incubated for 2 hours
C 0 5 ml dil of normal serum + 0 5 ml phage suspension + 0 2 ml heated RP Incubated for 4 hours
E 0 5 ml dil of normal serum + 0 5 ml phage suspension + 0 2 ml heated serum Incubated for 2 hours
G 0 5 ml dil of normal serum + 0 5 ml phage suspension + 0 05 ml heated RP Incubated for 4 hours

For further experimental details see text to Fig 2

prior to the incubation for two (curve C) and four hours (curve D) Curve E represents the phage inactivation reached after two hours when 0 06 ml RP was added to duplicate reaction mixtures before incubation It appears from C and D that the phage inactivating process is not definitely inhibited by RP after two hours The inactivation was found to proceed but at a retarded rate compared with the reaction velocity in the untreated reaction mixtures (A and B) Contrary to this delaying effect the lower dose of RP proved to enhance the phage inactivation considerably (F as opposed to A) About the same enhancement was achieved when 0 03 ml RP per ml reaction mixture was added

The enhancing action of a low dose (0 05 ml per ml reaction mixture) of RP and heated serum was compared in another experiment In an untreated reaction mixture, native serum (A O) diluted 1 22 4 was found to inactivate 50 per cent of the phages during two hours at 37° C The corresponding dilution was 1 25 0 and 1 48 8 respectively when heated serum and RP were added Thus the enhancing action of RP was much more marked

The influence of RP heated at 56° C for 30 minutes was further studied in experiments illustrated in Fig 8 Curves A and B represent the regular course of inactivation after two and four hours at 37° C in mixtures of phage and serial dilutions of native serum when no RP

appreciably affect the properdin activity as measured by the zymosan method

A salient characteristic of the effect of heated serum on the neutralization reaction is the occurrence of critical ratios between the *concentrations of heated and native serum* Even small variations in these ratios were found to cause considerable changes in the progress of neutralization, varying from enhancement to inhibition (cf Table 2 and Fig 3) The sensitivity of the neutralization reaction to small variations in molarity should be recalled in this connection It can, however, be concluded that the very small variations in the concentrations of fresh and treated serum able to influence the neutralization did not sufficiently change the molarity

The addition of RP allows the phage inactivation to proceed at high concentrations of C' and undefined serum factors, insofar as these factors were not adsorbed together with properdin by zymosan It was revealed by the present experiments that this addition did not lessen the fraction of phages resisting further inactivation by increasing concentrations of native serum On the contrary, *a lower level of inactivation* was reached after the addition of high concentrations of RP In these cases the inactivation gradually increased with the concentration of normal serum to the level obtained when no RP was present The increased phage inactivation at low concentrations of native sera after the addition of RP may indicate that the zymosan—adsorbable factors in the sera tested were not limiting Due to the complexity of the reaction system other explanations may, however, be conceivable The increased inactivation at low concentrations of fresh serum and the decreased inactivation at high concentrations contributed to a straightening of the sigmoid inactivation curve regularly obtained when RP was not added

Low concentrations of unheated RP were found to augment the inactivation This effect may be explained by the addition of limiting factors present *in RP For example, with the lowest RP concentration used, about 1 hemolytic units were added to each ml of reaction mixture* The concentration of RP added may thus be high enough to secure an excess of limiting factors whereas the amount of serum factors antagonistic to the neutralization reaction decreases The enhancing effect of low concentrations of RP may also be ascribed to the same phenomenon as makes low concentrations of heated serum enhancing

As reported above, the inhibiting effect appearing at certain ratios between RP and fresh serum was increased when RP was pre-heated at temperatures *able to inactivate the complement activity The effect* of heated RP was, however, stronger than that of heated serum though the resulting neutralization curves were similar in shape Heated serum and RP may influence the virus neutralization reaction by serum components originally present or released or formed as a result of the heating

Barlow et al (1958) found microgram quantities of bacterial and

animal polysaccharides and lipopolysaccharides to inhibit the phage neutralization reaction. They suggested that the inhibition by high concentrations of purified properdin and RP might be caused by soluble products from zymosan. Later *Landy et al.* (1958) demonstrated the inhibiting effect of polysaccharides to be a linear function of the dose. In the present investigation the effect of RP was found to switch from inhibition to augmentation on mere threefold dilution. These observations suggest that the inhibition by RP is probably not due to residual traces of zymosan products.

Neisser & Wechsberg (1901) observed an enhancing effect of heated specific immune serum diluted below the level of detectable autonomous effect on the bactericidal action of normal rabbit and goat sera. Larger amounts of immune serum abolished the bactericidal activity. This observation has later been confirmed by several investigators. The effect of phage antiserum on the neutralization brought about by normal serum has not been directly studied to the knowledge of the present author. However *Hershey & Bronfenbrenner* (1947) found that addition of complement to T2 sensitized with antibody, caused a considerable neutralization (it is not evident from the abstract referred to which form of complement was used). On the other hand complement under certain conditions inhibited the neutralization by immune serum particularly of T1. As mentioned above "normal antibodies" seem to be essential to the antiviral effect of normal serum. The influence of heated and zymosan adsorbed serum on the neutralization of T2 by normal serum may thus be analogous to the Neisser—Wechsberg phenomenon. This conclusion agrees well with the observations of *Maaløe* (1946) concerning the bactericidal effect of normal serum. Maaløe showed that subdetectable amounts of specific agglutinins in serum promoted the bactericidal effect of normal human serum while high concentrations had the opposite effect. He was also able to demonstrate that heated normal serum exercised an inhibiting action on the bactericidal effect of fresh serum. Owing to the similarity of the inhibitory action of immune serum and heated normal serum Maaløe considered the inhibitory normal substances to be identical with normal antibodies.

If the influence of RP and heated serum on the virus neutralizing action of normal serum is to be considered as analogous to the Neisser-Wechsberg phenomenon it would imply that the causative factor is originally present in fresh serum. The question why the neutralization inhibiting effect increases when the added serum reagents are exposed to higher temperatures must then be answered. The increasing effect within the temperature range 50°–64° C may be explained by the elimination of thermolabile antagonists or by the additional effect of factors formed at these temperatures. Further work is in progress to elucidate this problem.

SUMMARY

At critical concentrations of fresh serum large amounts of heated homologous serum or zymosan adsorbed serum exercised an inhibiting and smaller amounts an enhancing effect on the neutralization of coli T2 bacteriophages The difference between the amounts causing inhibition and enhancement was small a threefold decrease in concentration caused the reversal of a markedly inhibitory action The findings are discussed as possibly analogous to the Neisser Wechsberg phenomenon

REFERENCES

1 *Barlow J L Van Vunakis Helen & Levine L* Studies of the inactivation of phage by the properdin system I Evidence for complement properdin and magnesium requirements J Immunol *80* 339 348 1958

2 *Hershey A D & Bronfenbrenner J* Effects of complement on the specific neutralization of bacteriophage Fed Proc *6* 428 1947

3 *Kabat E A & Mayer M M* Experimental Immunochemistry C C Thomas Springfield Ill p 112 1948

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III Reactivation of inactivated coli T2 phages Acta path et microbiol scandinav 52 82 1961 c

IV Decay of the coli T2 phage inactivating capacity at various temperatures Acta path et microbiol scandinav 52 98 1961 d

6 *Landy M Trapani R J & Shear M J* Interference of endotoxic polysaccharides with inactivation of coliphage by human serum Brit J Exp Pathol *39* 618 630 1958

7 *Maaløe O* On the relation between alexin & opsonin Thesis Munksgaard Copenhagen 1946

8 *Neisser M & Wechsberg F* Ueber die Wirkungsart bactericider Sera Munch Med Wochenschrift *48* 697 700 1901

9 *Pernis B & Turri M* Osservazioni sul dosaggio della properdina mediante la neutralizzazione del batteriofago Boll Ist Sieroter Milan *37* 414 424 1958

10 *Pillemer L Blum L Lepow I H Wurz L & Todd E W* The properdin system and immunity III The zymosan assay of properdin J Exp Med *103* 1 13 1956

FROM THE NATIONAL BACTERIOLOGICAL LABORATORY, STOCKHOLM, AND
THE MICROBIOLOGICAL DEPARTMENTS OF THE MUNICIPAL LABORATORIES,
GOTHENBURG

STUDIES ON THE INACTIVATION OF BACTERIAL VIRUSES BY NORMAL HUMAN SERUM[1]

6. Comparative Studies of Various Phages and of Individual Sera of Human and Animal Origin

By

LARS OLOF KALLINGS[2]

Received 16.i.61

Studies on the kinetics and other characteristics of the inactivation of coli T2 phages have been presented earlier (*Kallings* 1961 a–e). The purpose of the experiments to be reported is to study to what extent other phages are neutralized by normal sera and to confirm the regular presence of the T2 neutralizing ability in human sera and also to study its occurrence in sera from various warm blooded animals. The scope of the study was restricted accordingly. The experiments were designed to allow of comparison between the inactivating capacity of different sera on the various phages. The sera were tested (a) on two groups of phages with known internal serological relations, i.e. the T series of coli phages and some staphylococcal phages, (b) on phages serolog...

MATERIAL AND METHODS

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... al. & Kallings 1959) the S. typhi ... (*Hammarström* 1948) and the

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[1] Aided by grants from the Swedish Medical Research Council.
[2] The skilful technical assistance of Mrs *Maud Lindberg* and Miss *Birgitta Sundolsson* is gratefully acknowledged.

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At critical concentrations of fresh serum large amounts of heated homologous serum or zymosan adsorbed serum exercised an inhibiting and smaller amounts an enhancing effect on the neutralization of coli T2 bacteriophages The difference between the amounts causing inhibition and enhancement was small, a threefold decrease in concentration caused the reversal of a markedly inhibitory action The findings are discussed as possibly analogous to the Neisser-Wechsberg phenomenon

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MATERIAL AND METHODS

The materials and m...
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F. coli B (T1 and T3–T7 were
Bacteriology, Karolinska Insti

al & *Kallings* 1955) the S. typhi
V (*Hammarström* 1948) and the

[1] Aided by grants from the Swedish Medical Research Council.

[2] The skillful technical assistance of Mrs *Maud Lindberg* and Miss *Birgitta Sundtisson* is gratefully acknowledged.

Staphylococcal phages (*Anderson & Williams* 1956 kindly supplied by Dr Anderson) were tested against their homologous strains in the inactivation tests

The staphylococcal phages (*Anderson & Williams* 1956) were kindly supplied by Dr G Wallmark the National Bacteriological Laboratory Stockholm who also isolated the KS 6 phage used (*Wallmark* 1954) For the inactivation test on staphylococcal phages the homologous bacterial strains were allowed to grow for five hours at 37° C in nutrient broth with 2 per cent glucose The nutrient agar described by *Anderson & Williams* (1956) for the typing of staphyl cocci was used as a base layer The same agar as described for the coli T2 inactivation test was used as top layer with the addition of CaCl to 0 4 per cent After the solidification of the top layer the plates were incubated at 37° C for three hours and then kept at room temperature overnight

The identity of the Salmonella Shigella and Staphylococcus phages were checked by their characteristic lytic activities towards the bacteria in the typing scheme

EXPERIMENTAL

Precision of the Assay of the Neutralization Titers Towards Coli T2 Phage

An analysis was made of the day to day variation in the titers of a given serum based on the means of 20 subsequent assays performed in duplicate An incubation time of two hours at 37° C was used In this case the titers were determined by three points in the semilogarithmic plot the percentages of inactivation obtained by three serial serum dilutions (1 5 fold steps) These values were found to vary around 81 46 and 11 per cent If the titers were calculated by interpolation between two adjacent points the standard deviation of the titers from various days was 8 1 per cent of the mean The corresponding standard deviation was 6 3 per cent if the titers were calculated on the basis of the best fitting straight line The mean slope of the straight lines was 1 98 ($\sigma = 0\ 17$)

Comparison between the Inactivation of Crude and Purified Coli T2r Phages

This comparison was undertaken partly to examine if there was any difference between preparations of coli T2r phages received from different sources and partly to find out if the kinetics of the neutralization reaction were influenced by the state of the stock suspension *i e* purified or crude lysate The comparison is of interest for the comparability of the present results with those of other workers Three virus stocks were compared (a) a crude lysate[1] (I) a purified preparation (II) and a crude lysate of phages propagated from the purified preparation (III) All stocks yielded more than 10^{10} plaques per ml The inactivation of I and II by six different sera according to the standard inactivation procedure showed about the same slopes for the linear part of the inactiva

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1 Kindly supplied by *Dr C C Heden* the Department of Bacteriology Karolinska Institutet Stockholm

2 Kindly supplied by *Dr J Barlow* the Division of Laboratories and Research New York State Department of Health Albany N Y

tion curves The phages of I, however, were reduced to a 10 per cent lower average level of survival at the higher serum concentrations (the persistent fraction) than phages of II The first mentioned phages were furthermore reduced to 50 per cent by an 18 per cent lower average concentration of the sera There was no difference between the inactivation of phages of I and III

Inactivation of Coli T1–T7 by different Sera

The inactivation of each phage by serial dilutions of five individual sera was examined in duplicate experiments according to the standard procedure The result is summarized in Tables 1 and 2 and in Fig 1 The sensitivity of the phages to the neutralizing serum activity expressed as the dilution inactivating 50 per cent of the phages was found to vary greatly The mean titers for each type of phage listed in Table 1 revealed a 12 5 fold difference between the most sensitive (T3) and the most resistant phage (T4) The individual serum titers for each phage are demonstrated in Fig 1 There was no obvious correlation between the serum titers obtained with the different phages not even within the serologically related groups Repeated neutralization tests showed about the same reproducibility for the assay of the neutralization of coli T1 and T3 T7 as for the neutralization of T2 As shown in Table 2 there was a difference between the data obtained for the mean titers of individual sera

TABLE 1
Inactivation of the T Series of Coli Phages by Serial Dilutions of Five Diferent Normal Sera

Phages (arranged in serological groups)	Serum dil causing 50% inactivation of the phages Mean	Inhibition by heated serum in % Mean
T 1	35 6	6
T 2	33 9	9
T 4	11 1	4
T 6	57 1	11
T 3	138 9	7
T 7	41 0	15
T 5	38 9	7

[illegible]

The phage inhibition brought about by serum heated at 56° C for 30 minutes and diluted 1 10 was of about the same magnitude for all phages (4 15 per cent, see Table 1) T7, however, was inhibited to 40 and 52 per cent by two of the heated sera



Fig 1.
Distribution of neutralization titers of five different normal human sera towards coli T1 T7 phages

TABLE 2
Inactivation of Coli T1 T7 Phages by Serial Dilutions of Five Different Normal Sera

Serum	Serum dil causing 50 % inactivation of the phages Mean	Inhibition by heated serum finally dil 1 10 Mean %
A	60 4	9
C	49 1	23
G	49 2	0
K	51 5	18
L	39 0	0

Inactivation of various Enterobacterial Phages by different Normal Sera

The inactivation of the Salmonella paratyphi B 0–1, the Salmonella typhi murium 39 and the Shigella sonnei V phages was tested in duplicate experiments using four different sera according to the standard inactivation procedure For comparison, the coli T2 phage was included in the experiments The result is presented in the same way as for the coli phages The mean data of the inactivation of the different types of phages are listed in Table 3 and of the individual sera in Table 4 On the whole the kinetics of the inactivation of the Salmonella and Shigella phages as revealed by the inactivation curves seemed to conform with the kinetics of coli T2 The reproducibility of the results seemed to be as good as with coli T2 phages As found with the T-phages, there was no correlation between the serum titers obtained with one and the same serum against the different phages (Fig 2) This is especially evident for serum B Coli T2 was not reduced to 50 per cent by serum B, further inactivation being inhibited at a level of 43 per cent even at high serum concentrations Irrespective of this, 0 1 phages were inactivated by serum B to a fairly high titer (1 50) In this case the progress of inactivation with increasing serum concentration stopped at the 75 per cent level of inactivation The phage inhibition caused by serum heated at 56° C for 30 minutes and finally diluted was found to vary with the phage and serum tested, from 8 to 30 per cent (mean values, see Tables 3 and 4)

TABLE 3

Inactivation of Various Enterobacterial Phages by Serial Dilutions of Four Different Normal Sera

Phage	Serum dil causing 50% inactivation of the phages Mean	Inhibition by heated serum finally dil 1 10 Mean %
E coli T 2	33.3	11
S pt B 0 1	46 5	19
S t m 39	25 0	9
Sh so V	62 0	27

Reaction mixtures 0 5 ml serum dilution + 0 5 ml phage suspension containing about 1 × 10⁴ plaque forming units Diluent 0 125 M barbiturate buffer, pH 7 4 ± 0 05 Mg^{++} added to 0 0025 M Incubated at 37° C for 2 hours

TABLE 4

Inactivation of Various Enterobacterial Phages by Serial Dilutions of Four Different Normal Sera

Serum	Serum dil causing 50% inactivation of the phages Mean	Inhibition by heated serum finally dil 1 10 Mean %
A	52 5	9
B	21 8	8
C	44 2	30
D	48 4	20

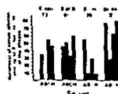


Fig 2

Distribution of neutralization titers of four different normal human sera towards various enterobacterial viruses

Inactivation of Staphylococcal Phages by different Normal Sera

The inactivating capacity of three different sera was compared on a [illegible] es represented [illegible] The sera were [illegible] activity was [illegible] dilution interval within which 50 per cent of the phages were neutralized (Table 5) In addition, the phage inhibiting effect of serum heated at 56° C for 30 minutes, finally diluted 1 10 and 1 50, was determined All experiments were performed in duplicate As illustrated by the table, four of the phages were reduced to 50 per cent by a final dilution of 1 50-1 250 of the different sera There was no



Fig 1
Distribution of neutralization titers of five different normal human sera towards coli T1–T7 phages

TABLE 2
Inactivation of Coli T1–T7 Phages by Serial Dilutions of Five Different Normal Sera

Serum	Serum dil causing 50 % inactivation of the phages Mean	Inhibition by heated serum finally dil 1 10 Mean %
A	60 4	9
C	49 1	23
G	49 2	0
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L	39 0	0

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of closely related phages The Vi phages routinely used for typing S typhi have all emerged through host adaptation of Vi phage II These variants have been shown to be either host induced modifications (phenotypic) or host range mutants (genotypic), or phenotypically modified host range mutants (*Anderson & Fraser* 1955) In addition to these Vi phage II variants, Vi phage I and IV were also tested The inactivation of the Vi phages by normal serum however, proved to be less demonstrable with the present technique than the inactivation of the other types of phages studied The purpose of the experiments was therefore restricted to show if a neutralization of the Vi-phages actually occurred and if there was any obvious difference in sensitivity to the serum action between the Vi phage II variants For this purpose ten different Vi phage II variants together with Vi phages I and IV were tested with dilutions of four different sera (Table 7)

TABLE 7

Per Cent Inactivation of Various S typhi Vi Phages by Four Normal Sera Diluted 1 10

	Phage		Normal serum G	M	N	O
Modification of Vi phage II	wild form of Vi phage II	A	75	61	69	
	phenotypic	C1	58	9	40	
	genotypic	D1	64			
	phenotypic of genotypic	D4	–	–	40	19
	genotypic	D5		–	35	–
	genotypic	D6			57	
	phenotypic	E1	59	69	39	–
	phenotypic	F1	2	13		–
	phenotypic of genotypic	F2				50
	genotypic	O		–		18
	Vi phage I		65	0		–
	Vi phage IV		72	59		–

Reaction mixtures 0 5 ml serum dilution + 0 5 ml phage suspension containing about 5×10^3 plaque forming units *Diluent 0 125 M barbiturate buffer* pH 7 4 ± 0 05 Mg added to 0 0025 M Reaction mixtures with serum G incubated for 4 hours at 37° C with the others for 2 hours – not tested

The sera were finally diluted 1 2, 1 5 1 10 1 20 and 1 100 and were incubated with the phages for two or four hours at 37° C The percentages of inactivation caused by serum diluted 1 10 are listed in the table As will be seen the inactivation caused by this high serum concentration was lower than the inactivation commonly observed with the other types of phages examined Thus the mean inactivation after two hours incubation was only about 39 per cent There was an obvious and *reproducible difference* between some of the phages, for example between Vi phage I and the other Vi phages (serum M) but also between the Vi phage II types Thus phage F1 being a host induced

correlation between the serum titers with the other three phages Serum A was found to contain thermostable inhibitors to all phages, serum B to five and serum C to three (Table 6) The mean inhibition caused by the heated sera possessing inhibitors in dilution 1 10 was 54 per cent, the corresponding inhibition in dilution 1.50 was 29 per cent High concentrations of native sera A and B did not inactivate phage 42 D whereas, after heating, the same sera did inhibit it For comparison of the inactivation curve of a staphylococcal phage with those of the other phages studied, the inactivation of phage KS 6 by serum C in 1 5-fold dilution steps was determined The resulting inactivation curve was similar to that previously described for T2

TABLE 5
Inactivation of Staphylococcal Phages by Three Different Sera (A B C)

Phage	Serological group of phage	Lytic group	Reciprocals of serum dil able to cause 50% inactivation of the phages			
			<5	5–50	50–250	250–1250
3 A	A	II		B C	A	
54	A	III			A B C	
70	A	III			A B C	
75	A	III			A B C	
52	B	I		C	A	B
42 D	F	IV	A B			C
KS 6	unclassified				A B C	

Reaction mixtures 0 5 ml serum dilution + 0 5 ml phage suspension containing about 5×10^3 plaque forming units Diluent 0 125 M barbiturate buffer pH 7 4 ± 0 05 Mg added to 0 0025 M Incubated at 37° C for 2 hours

TABLE 6
Inhibition of Staphylococcal Phages by Three Different Sera (A B C) Heated at 56° C for 30 Minutes

Phage	Final dil 1 10 of sera			Final dil 1 50 of sera		
	A	B	C	A	B	C
3 A	+		+			+
54	+			+		
70	+	+	—	+	+	
75	+	+	+	+		+
52	+	+		+	+	—
42 D	+	+	+	+	+	+
KS 6	+	+		+	+	

+ Inhibiting effect — No effect

Inactivation of Salmonella typhi Vi-Phages by different Normal Sera

The S typhi Vi phages were selected for study as they offered the unique opportunity of comparing the neutralization by normal serum

cent of the sera had titers between 10 and 30 The hemolytic complement activity in the reaction mixtures was estimated according to a standard procedure (*Kallings* 1961 a) to reveal sera with marked anti complementary effect No such sera were present The hemolytic activity was found to vary between 100 and 300 hemolytic units

The presence of heat stable inhibitors against T2 in normal human sera was reported in a previous paper (*Kallings 1961 d*)

In *Table 8* the reported distribution of the neutralization titers in human sera is compared with the results of other workers using a similar technique As will be seen the titers reported by *Fyquem et al* and the present author are somewhat higher than those reported by others

TABLE 8

Variations in the Coli T2 Phage Neutralizing Activity in Human Sera According to Different Reports

Author	Number of sera	Range of titers (PhN 50)	Remarks
Kallings	94	1-46	Mean titer about 15 84 per cent of the titers 10-30
Barlow et al (1958)	119	1-70	Majority of titers 6-20
Lernis & Turri (1958)	40	3-23	Mean titer about 9
Fyquem et al (1958)		15-50	85 per cent of the titers 18-33
Laurell & Reyn (1959)	86	2-46	Mean titer about 7

Inactivation of Coli T2 Phages by Various Animal Sera

The T2 inactivating ability of sera from 11 different animal species was tested in duplicate according to the standard procedure (with two fold dilution steps instead of 1 5 fold steps) The number of sera tested for each species is listed in Table 9 The blood specimens were handled as described for the collection of specimens from humans (*Kallings* 1961 a) As the neutralizing activity of the sera from some species was found to be low all mixtures of phage and serum were incubated for 4 hours at 37° C A phage inactivating effect was exerted with 9 of the species (Table 9) No inactivation was demonstrated with rabbit or mouse sera The titers appeared to vary considerably from species to species bovine sera exhibited the strongest effect guinea pig and sheep sera the weakest The titers of most human sera determined with the same reaction time are likely to fall between the values of monkey and fowl in the table Due to the limited number of animal sera tested no general conclusions can of course be drawn concerning the characteristic titer levels of each species

The general course of the inactivation curves was similar to that caused by human serum Typical curves for each species are depicted in Fig 4 (1 5 fold dilution steps) These curves are based on the inactiva

modification of phage A, proved to be more resistant to the serum action than phage A and other phenotypic variants. Phage F2 differed in sensitivity from phage D4, both being phenotypic variants of host range mutants.

An inhibiting effect after heating the sera at 56° C for 30 minutes was demonstrated only against the Vi-phage II type A and Vi-phage IV, the average inhibition by serum diluted 1:10 being 38 and 67 per cent respectively.

To sum up, these experiments revealed firstly that the Vi-phages could be inactivated by normal serum though the effect was weak. Secondly it appeared that the inactivation could vary both with the sera and the Vi-phages tested in spite of the very subtle differences between these phages. As mentioned above, the differences between the various adaptations of Vi-phage II cannot be distinguished by conventional serological methods.

Distribution of Neutralization Titers towards Coli T2 Phage in Sera from Healthy Humans [1]

Blood was drawn from 94 apparently healthy donors[2] and was handled as described in a previous paper (*Kallings* 1961 a). The sera were tested for T2 neutralizing activity according to the standard procedure. To allow of comparison with the results reported by other workers the mixtures were incubated at 37° C for one hour instead of the two hours ordinarily used by the present author. The distribution of the titers is presented in Fig. 3. As seen there was a roughly normal distribution. The geometric mean titer was found to be 15.2, 84 per

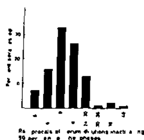


Fig. 3

Distribution of neutralization titers of normal sera from 94 healthy humans towards coli T2 phages.

Reaction mixtures: 0.5 ml serum dilution + 0.5 ml phage suspension containing about 1×10^4 plaque forming units. Diluent: 0.125 M barbiturate buffer pH 7.4 ± 0.05. Mg added to 0.0025 M. Incubated at 37° C for one hour.

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[1] A preliminary report was read at the 1959 Scandinavian Congress of Pathology and Microbiology. Published in Acta path. et microbiol. scandinav. Suppl. 144: 207, 1961.

[2] Fresh blood specimens were kindly supplied by *Dr. B. Gullbring* from the blood bank, Karolinska Sjukhuset, Stockholm.

inhibitors or anti complementary factors 0 1 ml native serum from each of these species was added to a series of one ml aliquots consisting of a mixture of phage and human serum (A A) finally diluted 1 33 3 For comparison 0 1 ml monkey serum or 0 1 ml buffer were added to other aliquots in the series The aliquots were incubated at 37° C for two hours and tested for residual phage activity according to the standard procedure As shown in Table 10 mouse serum totally inhibited the neutralization reaction Rabbit and sheep sera caused a partial inhibition whereas the addition of monkey serum did not decrease the inactivation in comparison with the inactivation reached when buffer was added The sera were only tested for anti complementary effect towards human complement 0 5 ml of the same human serum in the same dilution as used above was mixed with 0 5 ml sensitized sheep erythrocytes (4 amboceptor units) and 0 1 ml of the buffer or the animal sera was added The erythrocytes were completely lysed irrespective of the type of sera added

TABLE 10

Inhibiting Effect of Sera from Various Animal Species on the Neutralization of Coli T2 Phage by Normal Human Serum

Sera added to the human serum phage mixture	Per cent inactivated phages
Mouse	0
Rabbit	34
Sheep	26
Monkey	72
Buffer	72

Except for rabbit the results agree with those of *Barlow et al* (1958) who found that unheated sera from cow monkey dog guinea pig and rabbit listed in order of inactivation power neutralized coli T2 phages in the same manner as did human sera Mouse sera were found to have little or no neutralizing activity and prevented the inactivating ability when added to human sera The slope of the inactivation c[illegible] of rab[illegible]
in [illegible]
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the [illegible] an sera They were not able to demonstrate any activity in rat sera

DISCUSSION

It is evident from the present systematical study and the earlier isolated observations of several authors (to be reviewed in a following paper *Kallings* 1961 f) that normal human serum regularly contains factors inactivating a wide variety of bacterial viruses In the experi

TABLE 9

Inactivation of Coli T2 Phage by Normal Serum from Various Animal Species

Species	Reciprocals of serum dilutions causing 50% inactivation of the phages		Number of sera tested	
	Geometric mean	Range	Individual	Pooled
Ox	87.7	76.9–100.0	2	
Monkey (*Cynomolgus*)	70.4	54.6–98.0	4	
Dog	58.5	51.3–66.7	3	
Fowl	44.1	37.0–52.6	2	
Cat	22.4			2
Rat	19.3	11.6–26.7		2 × 5
Horse	18.3	13.9–23.8	4	
Guinea pig	17.1	12.2–32.1	4	
Sheep	< 14.6	< 10–25.6	4	
Rabbit	< 10.0	10	4	
Mouse	< 10.0	10		2 × 6

Reaction mixtures: 0.5 ml serum dilution + 0.5 ml phage suspension containing about 1×10^4 plaque forming units. Diluent: 0.125 M barbiturate buffer pH 7.4 ± 0.05, Mg added to 0.0025 M. Incubated at 37° C for 4 hours.

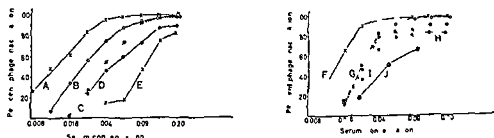


Fig. 4

Inactivation of coli T2 phages by normal sera from various animal species

Abscissa: Arithmetical values of serum concentrations on a logarithmic scale. Curve A: ox; B: monkey; C: horse; D: cat; E: sheep; F: dog; G: fowl; H: human; I: guinea pig; J: rat serum. Guinea pig and rat serum incubated for 4 hours at 37° C, the others for 2 hours at 37° C. All sera are not identical to those tested in Table 9.

tion reached after two or four hours incubation is indicated in the figure. High concentrations of sera from ox, monkey, dog and fowl were found to inactivate 99–100 per cent of the phages in contrast to the large persistent fractions regularly occurring with human sera. All sera were tested for heat stable inhibitors (56° C for 30 minutes), the final serum dilution 1 : 10 being incubated with phages at 37° C for the same time and at the same temperature as the native sera. The mean of 60 observations compared to the mean of 40 corresponding broth controls revealed an inhibition of the phage activity in the heated sera of 3.9 per cent.

In order to elucidate whether the absent or weak phage neutralizing effect of mouse, rabbit or sheep serum might be due to the presence of

antigenic stimulus by naturally occurring phages (*e g* to bacteria in the intestines), whether a heterogenetic antibody or whether a preformed specific antibody according to the theories of *Jerne* (1955) Several of the phages tested above are highly specific to bacteria which are not known to be so common that all individuals in the community could be expected to come into contact with them Several of these particular phages are pure laboratory products or anyway supposed to be It is not known, however, to what extent these phages share antigenic properties with phages to bacteria commonly residing in man and animal The presence of coli T2 neutralizing activity in sera from germ free guinea pigs (*Toussaint & Muschel* 1959) does not suggest the presence of antibodies formed as a response to a specific stimulus Due to the theoretical importance of the latter observation the present author tested some sera[1] from germ free rats for T2 neutralizing activity Preliminary experiments revealed a demonstrable activity (50 per cent inactivation of the phages after four hours at 37° C with serum diluted 1 11–1 25)

SUMMARY

The neutralizing activities of several normal sera on 29 enterobacterial (E coli T1–T7, Salmonella typhi Vi, S paratyphi B, S typhi murium and Shigella sonnei) and staphylococcal phages have been compared

The activities were found to vary with the sera as well as with the various phages There was no correlation between the activities of a given serum towards the various phages and the corresponding activities of another serum

The distribution of neutralization titers towards coli T2 phages in 94 human sera and in sera from 11 animal species was investigated

The results are discussed in relation to the possible origin of the factors determining the specificity of the phage neutralizing effect of normal serum

REFERENCES

1 *Anderson E S & Fraser A* The influence of the factors determining Vi type specificity in Salmonella typhi on the adaptation of Vi phage II J gen microbiol 13 519 532 1955

2 *Anderson E S & Williams R E O* Bacteriophage

3 *Barlo*

4 *Co*

[1] Generously supplied by *Prof B Gustafsson* Karolinska Institutet Stockholm

ments reported above it was found that the inactivation curves of most of the different phages were similar to those of the coli T2 phages as was also the thermolability of the serum activity. These findings are suggestive of a common system in the sera causing phage neutralization The sensitivity of a given phage towards various sera was, however, found to vary without any obvious similarity to the corresponding sensitivity of other phages The latter observations are in accordance with those reported by *Cowan* (1958) concerning the inactivation of coli T2, T6 and T 7 phages by several human sera The varying effect of a serum on different phages is confirmed by the observations of *Toussaint & Muschel* (1959) They found that absorption of normal human sera with E coli bacteria coated with phage T2 or T5 inhibited the phage neutralizing effect towards the homologous phage but not towards heterologous phages in the T-series Absorption with E coli cells alone resulted in a two or threefold reduction of properdin and a slight reduction of the hemolytic complement activity but only in an insignificant decrease in the coli T2 neutralization titer Attachment of T2 phages to the bacteria, though removing the T2 neutralizing ability, did not further decrease the properdin titer, the complement activity still being high Thus specific serum factors seem to participate in the phage inactivation Varying levels of these factors in different sera may explain the absence of a correlation between the activities of a serum towards various phages Analogous findings have been reported by *Osawa & Muschel* (1960) concerning the specific absorption of factors necessary for the bactericidal effect of normal serum towards Shigella bacteria

In the experiments referred to above, *Toussaint & Muschel* found that serum absorbed with T2 had about 50 per cent of the original neutralizing activity against phage T4 and T6, both serologically related to T2, whereas the activity against serologically unrelated T-phages was only slightly reduced As cited by *Laurell & Reyn* (1959), *Barlow et al* comparing the inactivating activities of various sera on some coli phages observed a constant ratio for 80 per cent of the sera tested with T2, T4, and T6 but variable ratios with T7 on the one hand and T2, T4 and T6 on the other In the present investigation no constant ratio against serologically related phages was obvious between the activities of the limited number of sera tested It is worthy of note that there was a difference between the inactivating capacity of a serum towards the various Vi-phage II variants, and even towards those representing phenotypic host range modification Experiments are in progress to investigate if the effect on these phages may be specifically absorbed in the same manner as with coli phages

In this connection, the question of the origin of the specific serum factor which seems to be necessary for the phage neutralization may be raised, whether it is a *conventional antibody*, with low avidity or present in subdetectable concentrations and formed as a result of an

FROM THE UNIVERSITY OF BERGEN SCHOOL OF MEDICINE THE GADE INSTITUTE DEPARTMENT OF MICROBIOLOGY AND DEPARTMENT OF PATHOLOGY BERGEN NORWAY

DISPERSION AND VIABILITY OF BCG AFTER INTRADERMAL INJECTION IN THE GUINEA PIG

2 *Pathological Findings*

By

TH M VOGELSANG and P WETTELAND

Received 19 xi 60

The problem of BCG dispersibility and survival time in the experimental host has been outlined in a previous communication (19) dealing with the bacteriological findings in guinea pigs within the first 17 weeks after intracutaneous administration of 0 1 mg bacilli In close agreement with the observations of earlier investigators (6 18 22) a rapid lymphogenous and haematogenous dispersion was observed In the course of the first week BCG had reached both the regional lymph nodes and the spleen and could be cultivated from the lymph nodes in decreasing numbers up to the 16th week after infection The present paper deals with the pathological findings observed in the same experiment

When normal guinea pigs were inoculated intracutaneously with BCG *Vorwald et al* (20) found that viable BCG organisms could sometimes be isolated from the lung and tracheobronchial lymph node eighteen months later Little evidence however is available about the persistence of living BCG organisms in man (8) and regarding the duration and localization of specific granulomas Originally the present investigation was incited by the interesting work of *Gormsen* (9), who in conjunction with medicolegal autopsies investigated the organs of 26 BCG vaccinated human beings between the ages of two months and 22 years BCG vaccination had been performed from 6 weeks to 12 years prior to death Gormsen concluded that the presence of granulomas in lymph nodes liver lungs spleen or kidney must at least up to two or three years after vaccination be regarded as a normal sequence of BCG vaccination Apparently BCG vaccination causes a generalized infection during the initial stages of which there is active multiplication of the bacilli which are disseminated throughout the body (8)

The primary reaction of the body to the tubercle bacillus varies considerably (3 15) depending on the species of animal (7 11 14) the

5 *Fyquem A Francq J C & Guyjot-Jeannin N* Comparative evaluation of properdin by means of inhibition of hemolysis and neutralization of the phage Meeting of the Reticuloendothelial Society, Rapallo August 1958
6 *Hammarstrom E* Phage typing of Shigella sonnei Acta med scandinav Suppl 223 1949
7 *Jerne N K* The natural selection theory of antibody formation Proc Natl Acad Sci *41* 849 857 1955
8 *Kallings L O* Studies on the inactivation of bacterial viruses by normal human serum
I Kinetics of the coli T2 phage inactivation at various serum concentrations Acta path et microbiol scandinav *51* 389 402 1961 a
II Effect of phage concentration temperature pH and molarity on the kinetics of the coli T2 phage inactivation Acta path et microbiol scandinav 52 71 81 1961 b
III Reactivation of inactivated coli T2 phages Acta path et microbiol scandinav 52 82 97, 1961 c
IV Decay of the coli T2 phage inactivating capacity at various temperatures Acta path et microbiol scandinav 52 98–112 1961 d
V Influence of heated and zymosan adsorbed serum on the inactivation of coli T2 phages To be published in Acta path et microbiol scandinav Vol 52 1961 e
9 *Kallings L O* Studies on the inactivation of bacterial viruses by normal human serum Thesis Stockholm 1961 f
10 *Laurell Anna Brita & Reyn Alice* Comparison between properdin titers obtained in the zymosan assay and in the bacteriophage neutralization assay I Investigation on normal human sera Acta path et microbiol scandinav 47 405 418 1959
11 *Lilleengen K* Typing of Salmonella typhi murium by means of bacteriophage Acta path et microbiol scandinav Suppl 77, 1948
12 *Osawa E & Muschel L H* The bactericidal action of normal serum and the properdin system *J Immunol 84 203 212 1960*
13 *Pernis B & Turri M* Osservazioni sul dosaggio della properdina mediante la neutralizzazione del batteriofago Boll Ist sieroter Milan 37 414 424 *1958*
14 *Thal E & Kallings L O* Zur Bestimmung des Genus Salmonella mit Hilfe eines Bakteriophagen Nord Vet Med 7 1063 1071 1955
15 *Toussaint A J & Muschel L H* Neutralization of bacteriophage by normal *serum Nature 183* 1825 1827 1959
16 *Wallmark G* Bacteriophage typing of Staphylococcus aureus pyogenes I A report of the method and some experimental results Acta path et microbiol scandinav 34 57 67 1954

FROM THE UNIVERSITY OF BERGEN SCHOOL OF MEDICINE THE GADE INSTITUTE DEPARTMENT OF MICROBIOLOGY AND DEPARTMENT OF PATHOLOGY BERGEN NORWAY

DISPERSION AND VIABILITY OF BCG AFTER INTRADERMAL INJECTION IN THE GUINEA PIG

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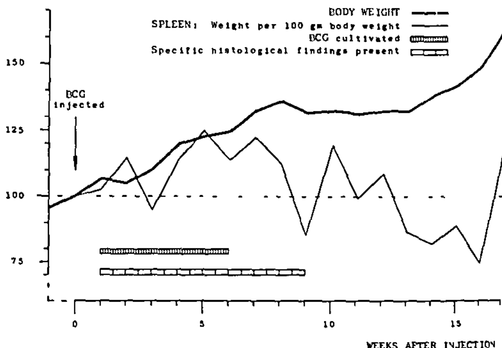


Fig 1
Body weight as percentage of average weight at BCG injection and weight of spleen per 100 g body weight as percentage of normal average The 100 per cent level is marked by the dotted line, and the intervals during which viable BCG units and specific histological changes in the spleen were found are indicated

size of dose (5, 18), the route of inoculation (8) and the virulence of the bacilli (10, 17) In a given species of animal, the tissue response may be different in various organs (14), and the developing allergy also plays its part in primary infection This different host-parasite relationship is reflected in the histological picture, which consequently may differ more or less from the classical description of the tubercle, and even completely lose its specificity following the local introduction of non specific antigens (21)

An attempt has therefore been made to analyze the various histological elements present in response to BCG in the guinea pig, comparing the site of injection, lymph nodes and spleen before giving a summary of the overall specific reaction in each of these organs

MATERIAL AND METHODS

Sixty seven normal guinea pigs were used 48 males and 19 females They were injected intracutaneously with 0 1 mg BCG on a depilated area in the right mamillary line at an equal distance from the right axilla and groin The details of the procedure are given in the first communication of this experimental series (19)

The local cutaneous reaction was read twice weekly until it had disappeared completely the degree of infiltration and the duration of necrotic lesions with or without suppuration being noted The animals were weighed once a week and the average body weight calculated as a percentage of the average weight at BCG injection

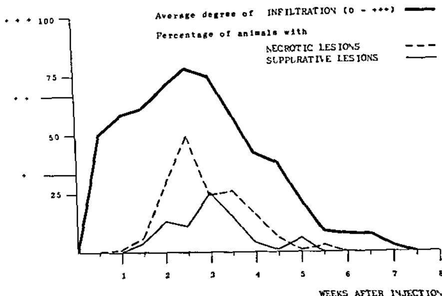


Fig 2
Local cutaneous reaction to BCG observed macroscopically twice weekly during the first 8 weeks of infection

Four animals were killed each week The site of injection, the inguinal axillary and para iliac lymph nodes and the spleen were removed and the latter weighed

The average weight of the spleen per 100 g body weight was calculated as a percentage of the average normal splenic weight, 6 male and 2 female guinea pigs serving as controls

One half of each organ was used for histological study, sections being stained with haematoxylin and eosin When specific tissue reactions persisted after the bacteriological cultures had become negative, sections were also stained according to the method of Z[illegible]

Every [illegible]
structur[illegible]
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were [illegible]

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RESULTS

The average body weight, calculated as a percentage of the average weight at the time of infection, showed a progressive increase interrupted by an interval with relatively constant values between the 9th and the 13th week (Fig 1)

The weight of the spleen as a percentage of average normal weight showed large variations, but the average value was, with a single ex-

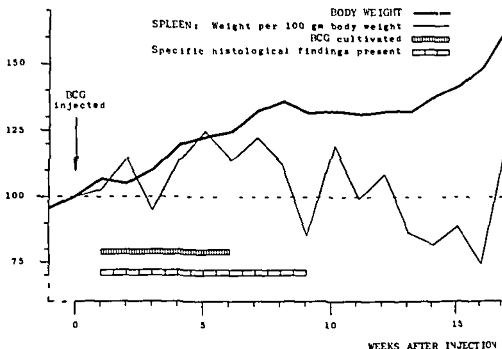


Fig 1

Body weight as percentage of average weight at BCG injection and weight of spleen per 100 g body weight as percentage of normal average The 100 per cent level is marked by the dotted line, and the intervals during which viable BCG units and specific histological changes in the spleen were found are indicated

size of dose (5, 18), the route of inoculation (8) and the virulence of the bacilli (10, 17) In a given species of animal, the tissue response may be different in various organs (14), and the developing allergy also plays its part in primary infection This different host-parasite relationship is reflected in the histological picture, which consequently may differ more or less from the classical description of the tubercle, and even completely lose its specificity following the local introduction of non-specific antigens (21)

An attempt has therefore been made to analyze the various histological elements present in response to BCG in the guinea pig, comparing the site of injection, lymph nodes and spleen, before giving a summary of the overall specific reaction in each of these organs

MATERIAL AND METHODS

Sixty seven normal guinea pigs were used 48 males and 19 females They were *injected intracutaneously with 0 1 mg BCG on a depilated area in the right* mammillary line at an equal distance from the right axilla and groin The details of the procedure are given in the first communication of this experimental series (19)

The local cutaneous reaction was read twice weekly until it had disappeared completely the degree of infiltration and the duration of necrotic lesions with or without suppuration being noted The animals were weighed once a week, and the average body weight calculated as a percentage of the average weight at *BCG*-injection

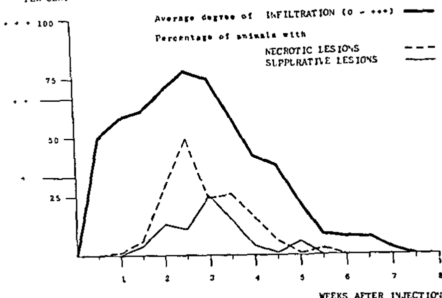


Fig 2
Local cutaneous reaction to BCG observed macroscopically twice weekly during the first 8 weeks of infection

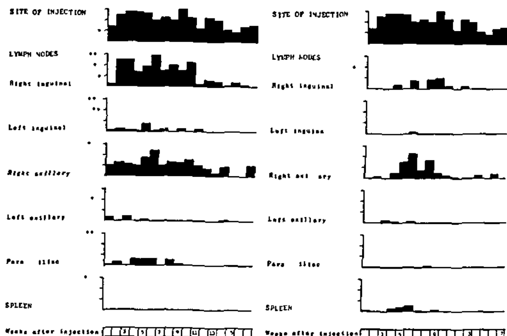
Four animals were killed each week The site of injection, the inguinal axillary and para iliac lymph nodes, and the spleen were removed and the latter weighed The average weight of the spleen per 100 g body weight was calculated as a percentage of the average normal splenic weight, 6 male and 2 female guinea pigs serving as controls

...ection was examined for the presence of ... structures Langhans' giant c... cellular structures (caseation ... were present in the follicles
In every section the abo... 0 +++ and the average deg... In addition the overall spec... nodes and spleen was record... logical findings (Figs 6 A D...

RESULTS

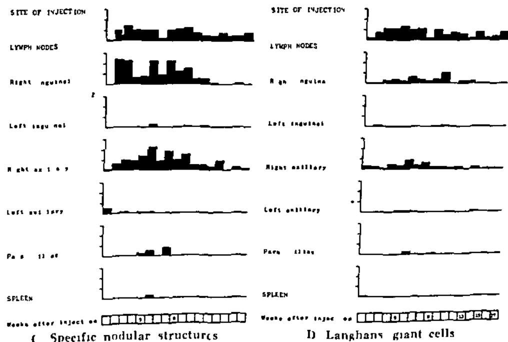
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A Epitheloid cells
Proliferation at the site of injection and in the pulp of lymph nodes and spleen

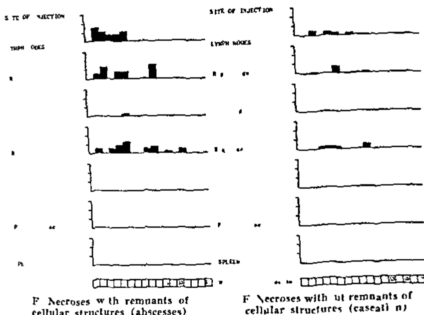
B Epitheloid cells
Proliferation at the site of injection and in the follicles of lymph nodes and spleen



C Specific nodular structures

D Langhans giant cells

Fig 3
Histograms representing histological findings



F Necroses w th remnants of cellular structures (abscesses)

F Necroses with ut remnants of cellular structures (caseati n)

Fig 3

ception above normal during the period of positive bacteriological and histological findings and mounted to a maximal increase of 25 per cent five weeks after infection (Fig 1)

The degree of local cutaneous infiltration increased rapidly and reached its average maximum 2½ weeks after injection (Fig 2) followed by a steady decline and final complete disappearance in the 8th week after which no trace except pale scars was left in any animal At the height of the infiltration one half of the animals presented necrotic lesions followed by suppurating ulcers in 25 per cent of the guinea pigs at the end of the third week

Histological Findings

At the site of injection the epitheloid tissue reaction was well developed one week after infection and nearly maximal in the third and fourth weeks after which a slow and inconstant decrease took place Specific alterations were still present at the end of the experiment Figs 3 a and b 4 b)

In the lymph nodes the epitheloid reaction was generally more marked and of slightly longer duration in the pulp than in the follicles and decidedly more pronounced in the right inguinal and axillary lymph nodes than in the other groups (Figs 3 a and b 5) The least reaction was observed in the group of left axillary lymph nodes In the spleen proliferation of epitheloid cells was scanty only observed within

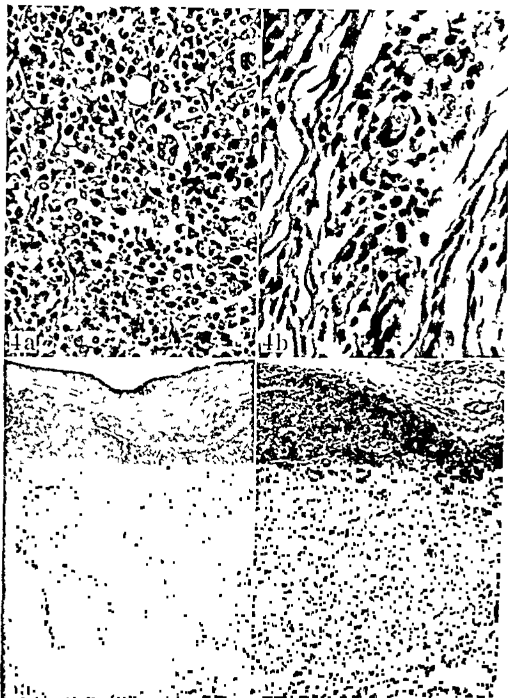


Fig 4

Site of injection

A Epitheloid cells (left), young giant cells (right) and incipient necrosis (bottom) Two weeks after infection Rich growth of BCG (H & E × 300)

B Tubercle in the corium 16 weeks after infection No growth of BCG (H & E × 420).

Fig 5

Right inguinal lymph nodes

A Large tubercles with central necrosis, massive capsular thickening (hilar region) Three weeks after infection Medium growth of BCG (H & E × 22)

B Pronounced tuberculous lesions present 10 weeks after infection Epitheloid and giant cells in wall of large abscess Scanty growth of BCG (H & E × 110)

the follicles, and not seen after the 9th week of infection (Figs 3 a and b)

The finding of typical tubercles, viz specific nodular structures, varied markedly in degree and duration, but was most pronounced locally and in the right inguinal and axillary lymph nodes (Fig 3 c) Typical tubercles were observed locally throughout the experiment Well developed nodular patterns developed more slowly, but were somewhat longer lasting in the right axillary nodes compared to the inguinals on the same side, in which the nodular structure had almost disappeared after the 13th week, while it was still present in the axillary nodes 17 weeks after immunization The reaction was very slight and inconstant regarding the nodes on the left side, but principally the same, nodules being observed up to the 14th week in the axillary nodes, but not after the 12th in the inguinal group The reaction was slightly more pronounced in the para iliac lymph nodes, but present over a shorter interval (5th to 8th week) In the spleen, specific nodules were only demonstrable in the 6th week after infection

The presence of Langhans' giant cells varied, as could be expected, with the finding of specific nodular patterns, most of the giant cells being found within nodules of mature tubercles (Fig 3 d)

Necrotic lesions containing masses of pyknotic cells or remnants of cellular structures (abscesses) were more frequently seen than in the classical picture of structureless caseation (Figs 3 e and f, 4 a, 5), but never observed in the left axillary and para-iliac groups of lymph nodes or in the spleen As usual, the findings were most marked locally and in the lymph nodes of the right side, subsiding earlier in the inguinal than in the axillary nodes, where cellular necrosis was present after 14 weeks

The total tuberculous reaction was, as might be expected, generally most pronounced and longest lasting at the site of injection (Fig 6 a), where the maximal response was observed after 3 weeks A slow but progressive decline then followed, with persistence of slight but definite specific alterations at the end of the experiment 17 weeks after infection

In sections of the local, right inguinal and axillary lymph nodes (Figs 6 b and c), the overall reaction was comparable in intensity to that observed at the site of injection during the first 10 weeks after infection, but not as constant, developing and decreasing more slowly in the right axillary nodes than the inguinal After the 10th week a relatively rapid regression of the local lymph node lesions took place, leaving no trace in the right inguinal group at the end of the experiment

Regarding the other groups of lymph nodes and the spleen (Figs 6 b, c and d), the total tissue reaction was slight, inconstant and of relatively short duration, disappearing between the 9th and 10th week of infection



Fig 4

Site of injection

A Epitheloid cells (left), young giant cells (right) and incipient necrosis (bottom) Two weeks after infection Rich growth of BCG (H & E × 300)

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B Pronounced tuberculous lesions present 10 weeks after infection Epitheloid and giant cells in wall of large abscess Scanty growth of BCG (H & E × 110)

After cessation of growth, specific lesions persisted in a total of 28 animals, mainly at the site of injection Acid fast bacilli or granules were, however, observed in 8 animals only, and in very scanty numbers With one exception, the acid-fast material was always found intracellularly These findings are summarized in Table 1, which also states the last week of positive cultures and presence of specific host reaction, respectively, for the various organs

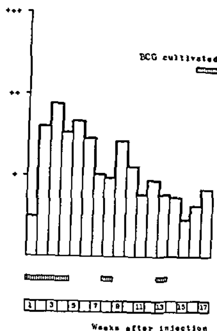
TABLE 1

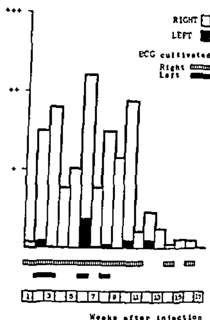
Presence of Acid Fast Bacilli or Granules in Persisting Culture Negative Lesions

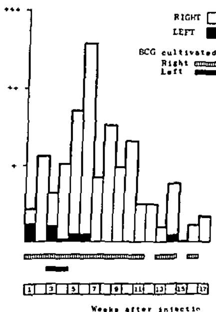
Organ		Week of last positive culture	Week of last tuberculous lesions	No of animals with culture negative lesions	Acid fast material			
					No of animals	Week	Findings	Phagocytosis
Site of injection		13th	17th	15	1	14th	Very few bacilli	Absent
					1	14th	Many granules	+
					1	15th	Few granules	+
					1	16th	Few granules	+
Lymph nodes	Rt ing	16th	16th	3	0			
	Lt ing	8th	12th	2	1	10th	Many granules	+
	Rt ax	16th	17th	3	1	12th	Few granules	+
	Lt ax	4th	14th	3	1	17th	Few granules	+
	Para iliac	12th	9th	0	0			
Spleen		6th	9th	2	1	9th	Very few bacilli	+

DISCUSSION

In the present experiments, a well developed specific tissue response to BCG was observed at the site of inoculation and in the local inguinal and axillary lymph nodes At the site of injection, specific alterations persisted much longer than the growth of BCG, and were still present at the end of the 17th week In the local lymph nodes, the tuberculous inflammation was most marked between the second and the tenth week after infection, lagging a little behind in the axillary nodes compared to the inguinal group During this period of well-marked histological alterations, growth was continuously obtainable from both groups of local nodes and during the last week the specific lesions trailed off as the growth became scanty and finally disappeared In the other groups of lymph nodes and in the spleen an equal parallelism was demonstrable between host reaction and growth, both, however, being here slight, inconstant and of comparatively short duration







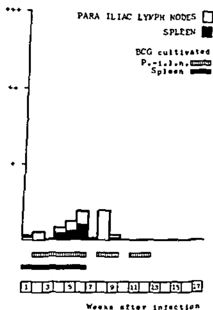


Fig 6

Histograms representing overall specific reaction The intervals with positive BCG cultures are indicated for comparison of histological and bacteriological findings

A Site of injection

B Right and left inguinal lymph nodes

C Right and left axillary lymph nodes

D Para iliac lymph nodes and spleen

the severe damage to tissue that leads to necrosis or caseation, and that the tubercle formed by BCG regresses completely and usually without fibrosis or calcification In the present series, calcification was never observed Fibrosis was sometimes present in local lymph nodes towards the end of the experiment 17 weeks after infection As a rule, however, the lesions had generally disappeared by this time, leaving no trace except at the site of injection and in the right axillary lymph nodes

Necrosis or caseation, however, could be seen in active lesions at the site of infection and in the local nodes, and the amount of tissue damage corresponded well to the simultaneous degree of growth A close parallelism could be expected, since greater numbers of bacilli will invariably produce more necrosis than small numbers within the same tissue, the extent of necrosis being dependent much more upon the number of bacilli present than upon the size or even the age of the tubercle (2) Judged by the present observations, the number of bacilli is probably as important as their virulence with regard to their capacity to produce necrosis or caseation

Thus, while the presence of tissue damage in primary infection apparently depends more upon the bacilli than the host, both seem to be important regarding the appearance of necrotic lesions as opposed to structureless caseation In our experiments the former cannot be fully explained as a precursor of the latter, since caseation occurred at approximately the height of necrosis, and subsided earlier in the local lymph nodes Cellular necrosis is the typical tissue damage caused by BCG in the guinea pig, not structureless caseation, and more or less nodular formations of epitheloid cells are generally observed more frequently than typical tubercles of classical description

Anderson (2) states generally that caseation is progressive so long as tubercle bacilli proliferate actively in the tissues and extension of the process continues to take place Necrosis and caseation coincided with growth in the local lymph nodes in our series, but not at the site of injection, where the majority of the caseating lesions were noted when the growth was markedly decreasing or had even disappeared

In order to produce a tuberculous lesion in the body, tubercle bacilli must grow within phagocytic host cells, but the capacity of the bacilli to survive and multiply appears to be related to their virulence (13) As the virulence of BCG is very low even for guinea pigs, the present scanty findings of intracellular acid fast bacillary remnants in only a few late lesions are in close agreement with this assumption

At the end of the present experiment, tuberculous alterations only persisted locally and within the right axillary lymph nodes, but were definitely about to disappear, confirming that the experimental tubercle may resolve, disappear completely, and leave no trace (3)

In the right inguinal nodes, disappearance of reaction coincided with cessation of growth. With the exception of these and the para-iliac lymph nodes, the histological response to BCG always persisted longer than the growth, and this discrepancy was most marked at the site of injection, where elimination of the bacilli was almost completed 4 weeks after injection (19).

These experimental findings support the observations made by *Adamson* (1) in human beings. He examined cervical, mediastinal and abdominal lymph nodes as well as tonsils and spleen from 67 patients, 23 of whom had died from tuberculosis, and found good agreement between the positive Lowenstein cultures and the histological finding of tuberculous lesions.

The transient loss of increase in body weight during the second half of the experiment might well be due to chance, the average weight being calculated on a constantly decreasing number of animals. It may also, however, owe its explanation to the intercurrent infections mentioned in the preceding paper (19). The weight of the spleen, being a fine indicator regarding stimulation of the reticulo-endothelial system, varied but showed on the whole a slight increase during the period of well-marked general findings. Definite conclusions cannot be drawn, however, as each average weight is based upon four animals only, allowing considerable chance variation.

The macroscopic observations of the local reaction corresponded well to statements of previous authors (4, 5) regarding comparable doses of BCG. As different strains of BCG have been shown to differ very little in virulence (4), the degree of the primary local reaction is mainly dependent upon the size of the infecting dose. Generally, the clinical course of the primary skin response to BCG-vaccine by intracutaneous injection in the guinea pig imitates pretty well the response to the same procedure in the uninfected human being (12), even though it has been doubted that the virulence of BCG is exactly the same for human beings as for guinea pigs (4).

Regarding the observed cellular response to BCG, that of the epitheloid cells is the most interesting, because these constitute the principal element in tuberculous inflammation. Considering the general tissue reaction to tubercle bacilli (3), it has been stressed that by the time the mononuclears become transformed into epitheloid cells the bacilli have undergone extensive destruction. This seems in our experiments to hold true for the BCG-infected guinea pig at the point of entrance of the bacilli, but not for the metastatic foci in lymph nodes and spleen, where the main epitheloid response corresponded fairly well to the period of maximal growth.

The aspects of non-caseating tuberculosis have been reviewed by *Pinner* (16). *Rosenthal* (17), working with intracardially injected guinea pigs, stressed that the most significant differences between tubercles formed by BCG and virulent bacilli are that BCG does not cause

16 *Pinner M* Noncaseating Tuberculosis An Analysis of the Literature Am Rev Tuberc *37* 690–728 1938

17 *Rosenthal S R* Focal and general tissue responses to an avirulent tubercle bacillus (BCG) Arch Path *22* 348 372 1936

18 *Soltys M A & Jennings A R* The dissemination of tubercle bacilli in experimental tuberculosis in the guinea pig Am Rev Tuberc *61* 399 406 1950

19 *Vogelsang T M & Wetteland P* Dispersion and viability of BCG after intradermal injection in the guinea pig 1 Bacteriological examinations Acta path et microbiol scandinav *48* 169–175 1960

20 [illegible] accination in [illegible]

21 [illegible] oid vaccine) [illegible] andinav *38* 466–480 1956

22 *Willis H S* Studies on tuberculous infection X The early dissemination of tubercle bacilli after intracutaneous inoculation of guinea pigs of first infection Am Rev Tuberc *11* 427 449 1925

CONCLUSIONS

Following the intracutaneous injection of 0 1 mg BCG in guinea pigs, a well-marked tuberculous tissue response developed at the site of injection and in the local inguinal and axillary lymph nodes Seventeen weeks after injection all growth had ceased, and the host response had disappeared or was trailing off in the local nodes while still persisting at the site of injection Both the histological reaction and the growth of BCG were slight, inconstant and of shorter duration for other groups of lymph nodes and the spleen

On the whole, the degree of growth reflected the specific histological activity, but the growth generally subsided some time before the lesions

The various histological elements in BCG inflammation are discussed, particularly the type of tissue damage and the patterns of epitheloid response

REFERENCES

1 *Adamson C -A* A bacteriological study of lymph nodes Analysis of postmortem specimens with particular reference to clinical serological and histo pathological findings pp 71–74 Esselte, Stockholm 1949

2 *Anderson W A D Pathology pp 238–242 C V Mosby, St Louis 1953*

3 *Boyd W* A textbook of pathology An introduction to medicine pp 161–172 Lea & Febiger, Philadelphia 1947

4 *Boe, J* Variations in the virulence of BCG Acta tuberc scandinav *22* 125 133 1948

5 *Böe J* Den optimale dose ved BCG vaksinasjon (The optimal dose of BCG) Nord med *42* 1255–1257, 1949

6 *Desbordes J Bonet Maury P & Deysine A* Application pratique des bactéries marquées Etude de la vitesse de dispersion du *Mycobacterium tuberculosis* marque au ^{32}P chez le cobaye Sort précose des bacilles en la présence ou en l absence d hyaluronidase Compt rend Soc de biol *147* 1212 1214 1953

7 *Feldman W H & Karlson A G* Experimental intracutaneous tuberculosis in *the white rat J Invest Dermat 3* 107 117 1940

8 *Fenner F* Bacteriological and Immunological Aspects of BCG Vaccination Bibliotheca Tuberculosea Fasc 5 Advances in Tuberculosis Research *4* 112 186 1951

9 *Gormsen H* On the occurrence of epitheloid cell granulomas in the organs of BCG vaccinated human beings Tr *XIth* Scandinav Congr *Path & Bac* teriol Aarhus 1955 Acta path et microbiol scandinav *Suppl 111* 117 120 1955

10 *Jensen K A & Bindslev G* Experimental Studies on the Development of Tuber *culous Infection in Allergic and Non Allergic Animals II Development* of Tuberculous Infection in the Lungs after Inhalation of Low virulent and High virulent Tubercle Bacilli Acta tuberc scandinav *11* 101 129 1937

11 *Lack C H* Chronic tuberculous infection in experimental animals Am Rev Tuberc & Pulm Dis *73* 378 389 1956

12 *Leider M & Sulzberger M B* Studies in the Allergy of Infection I Responses of the Skin to BCG Vaccination in Various Categories of Tuberculin Sen sitivity J Invest Dermat *13* 249 264 1949

13 *Mackaness G B* Relationship between host cell and parasite in tuberculosis Brit M *Bull 10* 100–104 1954

14 [illegible] *& Kiser J S* A Histological Study of the Mice Experimentally Infected with Bacilli Surg *12* 367 429 1954

15 *Pagel W* Pathologie der experimentellen Meerschweinchentuberkulose Zen tralbl f d ges Tuberk Forsch 37 305 315 1932

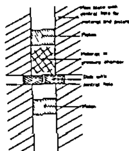


Fig 1
Simplified section through the X press

No particular method for evaluating the degree of disintegration is markedly superior to others They often complement each other, so in this investigation, a number of estimation methods were used in parallel, *viz*, viable count, total count, extinction at 600 mμ, turbidity, viscosity, and light- and electronmicroscopy and -photography The amount of nitrogen solubilized by the disintegration process has also been determined This multiple characterization of the material obtained may help understanding the disintegration process

MATERIALS AND METHODS

Cultivation of test organisms was performed as described earlier in a synthetic medium and harvested in the logarithmic phase of growth (*Edebo* 1960) The cells were washed and suspended in approximately 0 07 M sodium citrate phosphate buf

and then the presses were cooled for another hour in order to attain the

the minimal pressure required for phase transitions at that temperature This is probably due to the low velocity of phase transition reactions at such low temperatures

Estimation of Disintegration

After th
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question When the time came to estimate the efficacy of disintegration, the samples were thawed and kept in an ice water

[1] Kindly supplied by Dr *H Markkula*

DEPARTMENT OF BACTERIOLOGY, KAROLINSKA INSTITUTET, STOCKHOLM 60, SWEDEN. HEAD: PROFESSOR B. MALMGREN

DISINTEGRATION BY FREEZE-PRESSING

I. *Effects on Bacteria*

By

L. Edebo

Received 6.iii.61

Disintegration of cells is an important step in releasing many substances for biochemical study or practical use. These substances are often of great biological interest, but since they are labile, the techniques used for breaking up the cells are of great importance. This is illustrated by the great number of methods that have been proposed, a variety which in itself suggests that a completely satisfactory technique is difficult to develop. For obvious reasons, disintegration at low temperature is of particular interest, and this study was undertaken in the course of a series of investigations concerned with such techniques.

Many different kinds of cells are disintegrated when forced in the *frozen* state *through a hole* some *millimeters in diameter in a novel* kind of press, the X-press (Fig. 1) (for details, see *Edebo* 1960). The flow of the frozen material through the hole is intimately connected with changes in the crystal structure of ice under high pressures (*Tammann* 1903, *Bridgman* 1912), but the details of the process is not yet clear. As a rule the disintegration is performed at —25° C, where a pressure of about 2000 atm. is required to cause a flow of material. This pressure is known to transform ice I into ice III, a process characterized by a volume reduction of about 20 per cent. This change has been shown to produce a modest disintegration of Escherichia coli B (*Edebo & Heden* 1960). Since the *receiving chamber of the X-press is an exact* image of the pressing chamber, the cells may be forced many times through the hole without uncoupling the apparatus. This is particularly valuable when the cells are hard to break, which is the case with some microorganisms.

The present investigation was performed in order to elucidate the phenomenon of disintegration of bacteria. A thorough examination of the disintegration products from *Escherichia coli B and Streptococcus* faecalis plus a comparison with a variety of other bacteria were carried out.

This work was made possible *by a grant from the Wallenberg Foundation.* The technical assistance provided by Miss *Ulla Spetz* is gratefully acknowledged.

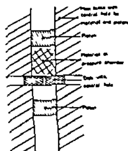


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No particular method for evaluating the degree of disintegration is markedly superior to others They often complement each other; so in this investigation, a number of estimation methods were used in parallel, viz, viable count, total count, extinction at 600 mμ, turbidity, viscosity, and light- and electronmicroscopy and -photography The amount of nitrogen solubilized by the disintegration process has also been determined This multiple characterization of the material obtained may help understanding the disintegration process

MATERIALS AND METHODS

Cultivation of test organisms was performed as described earlier in a synthetic



... is greater than the minimal pressure required for phase transitions at that temperature This is probably due to the low velocity of phase transition reactions at such low temperatures

Estimation of Disintegration

After the pressing operation the samples were transferred without thawing to a −25° C freeze box and kept there for a few days The time the pressed samples were kept in the freeze box before thawing did not influence the number of viable cells appreciably within the time range in question When the time came to estimate the efficacy of disintegration the samples were thawed and kept in an ice water

[1] Kindly supplied by Dr *H Markkula*

bath for one hour to allow time for solution of the protoplasm All manipulations except viscosity measurements were performed within four hours and the materials were held in the ice water bath throughout The viscosity, however, was measured on non-centrifuged samples immediately after thawing

Viable counts were obtained after serial ten fold dilutions in nutrient broth followed by plating of 0 1 ml aliquots on nutrient agar plates, and in the case of E coli also on Endo's agar Three plates were inoculated for each dilution, and plates with 30 to 300 colonies were counted after overnight incubation at 37° C The difference between the highest and the lowest count of the three plates very rarely exceeded 20 per cent

Total counts were made by use of phase contrast microscopy and the aid of a Buerker counting chamber (depth 0 01 mm)

The optical density (*extinction*) and light scattering power (*turbidity*) of the non-centrifuged material were determined in a Beckman DU spectrophotometer at 600 mμ and in a nephelometer (B Lange, Berlin), respectively The nephelometer used had its linear semilogarithmic scale range between 25 and 85 units (Fig 20) When at the dilution suitable for most of the suspensions of a series, one suspension was so dense that the turbidity fell beyond the linear part of the scale more comparable turbidity values were obtained by extrapolation (Fig 20) of a more diluted suspension These values are shown in brackets in Table 1, 2 and 3

After centrifugation (35 000 RPM for 90 min in the 40 rotor of the Spinco Model L ultracentrifuge, R_{av} = 81,000 x g) the *nitrogen* content (Kjeldahl) of the supernatants was determined The nitrogen content of the non-centrifuged bacterial suspension is given in brackets (Table 1, 2) The extinction at 260 and 280 mμ of the supernatant was also determined Since this method was not reproducible these data are omitted

Smears were prepared from the pellets, and the presence of distinctly stained cells was noted after staining with dilute fuchsin (Gram-negative bacteria), Gram's stain (Gram positive cells) or Hallberg's Nachtblau stain (Hallberg 1946) in the case of Mycobacterium phlei The disintegration was estimated as complete (+++++) 90–99 per cent (++++), 70–90 per cent (+++), 40 70 per cent (++), 10 40 per cent (+) or absent (—)

In order to minimize enzyme activity the *viscosity* of the noncentrifuged disintegrated materials (diluted 1 5 with citrate-phosphate buffer) was determined with an Ostwald viscosimeter immersed in an ice-water bath in a cold room (+ 3° C) immediately after thawing

The viscosity is listed as the specific viscosity (η_{sp}, *Jirgensons* 1958)

$$\eta_{sp} = \frac{t_1 - t_0}{t_0}$$

(t_0 = time for plain citrate phosphate buffer and t_1 = time required for the sample to flow through the capillary of the viscosimeter)

The disintegrated material was also *photographed* in a Zeiss photomicroscope with phase contrast attachment

Specimens for electron microscopy were prepared after several washings by an air drying method (*Edebo & Cedergren* 1961) They were then shadowed with chromium and photographed in an Akashi Tronscope TRS

RESULTS

Escherichia coli

When E coli are pressed (Table 1 and Fig 2 illustrate the same experiments), the total and viable count, the extinction at 600 mμ, and the turbidity are decreased As long as total counts can be made, they parallel the viable counts The untreated bacteria give a higher count on Difco agar than on Endo's agar, but all pressed samples yield higher counts on Endo's agar The estimated cell reduction is less in fuchsin stained smears than suggested by the total and viable counts

TABLE 1

Disintegration of E. coli

Nephelometric value in brackets calculated by extrapolation from dilution 1:100 with the aid of Fig. 20. The column with mg N/ml usually shows the nitrogen contents of the supernatants but the value in brackets shows the nitrogen content of the whole suspension.

Number of passages	Viable count (bact/ml)		Total count (bact/ml)	Fuchsin stained smears	Extinction 600 mμ Dilution		Nephelometric value Dilution		Specific viscosity η_{sp} dil 1:5	mg N/ml (supern.)
	Difco agar	Endo's agar			1:10	1:100	1:10	1:100		
0	3.6×10^{10}	2.8×10^{10}	1.3×10^{11}		∞	0.90	96 (145)	73	0.10	0.39 (5.07)
1	9.2×10^{9}	1.5×10^{9}	1.5×10^{10}	++	0.96	0.136	76	18	1.12 – 0.58	3.42
2	1.5×10^{9}	2.6×10^{9}	7.8×10^{9}	++	0.82	0.103	64	10	0.30	3.68
3	4.4×10^{7}	9.3×10^{7}	9.3×10^{8}	+++	0.50	0.076	47	8	0.27	3.92
5	5.2×10^{6}	1.1×10^{7}	$< 10^{8}$	++++	0.51	0.062	48	8	0.26	3.81
9	1.8×10^{5}	3.6×10^{5}	$< 10^{8}$	++++	0.54	0.060	46	14	0.18	3.81

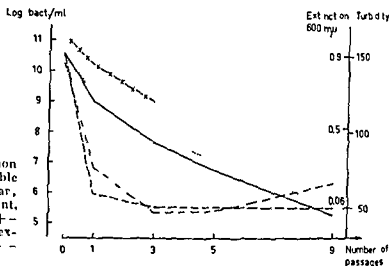


Fig 2 Disintegration of E coli (—— viable count, nutrient agar, ... viable count, Endo's agar, -+-+- total count, ---- extinction 600 mμ, - - - turbidity)

Figs 3–5
Phase contrast microphotographs of E coli (3) Non disintegrated E coli (4) Bacteria pressed once (5) Bacteria pressed nine times After one pressing many cell walls are seen but after nine pressing no particular structures are found apart from a clumping



Figs 6 10

TABLE 2

Disintegration of Str faecalis

Nephelometric value in brackets calculated by extrapolation from dilution 1 100 with the aid of Fig 20 The column with mg N/ml usually shows the nitrogen *contents of the supernatants* but the value in brackets shows the nitrogen content of the whole suspension

Number of passages	Viable count (bact ml)	Total count (bact ml)	Gram stained smears	Extinction 600 mμ Dil 1 25	Nephelometric value Dilution		Specific viscosity η_{sp} Dil 1 5	mg N ml (supern)
					1 10	1 100		
0	1 5 × 10^{10}	1 1 × 10^{11}	-	1 38	97 (119)	47	0 03	0 30 (2 91)
1	2 5 × 10^{8}	2 3 × 10^{10}	++	0 69	83	18	0 21	1 18
3	1 3 × 10^{7}	1 4 × 10^{10}	++	0 46	64	11	0 17	1 64
5	1 5 × 10^{6}	7 3 × 10^{9}	+++	0 34	48	7	0 17	1 79
9	3 4 × 10^{4}	1 8 × 10^{9}	++++	0 18	26	5	0 14	1 93

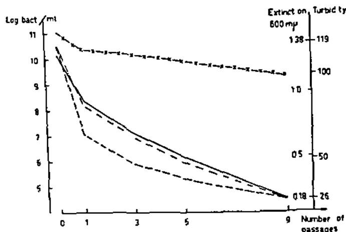


Fig 11
Disintegration of Str faecalis (—— viable count nutrient agar –+–+ +– total count –– extinction 600 mμ – – – turbidity)

The first pressing causes a considerable drop in the extinction at 600 mμ of the suspension The successive pressings cause further decreases in extinction so that it asymptotically approaches a limit value The turbidity, however, is decreased only by the first three pressings and then increases

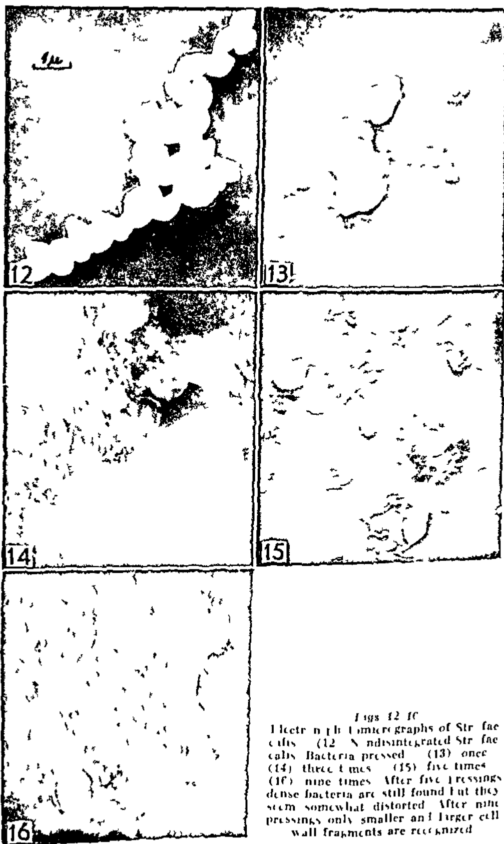
The material pressed once shows a high viscosity, but the viscosity of materials pressed many times is reduced The soluble nitrogen remaining in solution after centrifugation is increased only by the first three pressings and then decreases slightly

After one pressing many *well defined* cell walls are found in the phase contrast and electron photomicrographs (Fig 4, 7) After a number of pressings however, the cell walls are disintegrated into smaller fragments (Fig 8 9) Aggregation of the cell walls also occurs (Fig 10) This is particularly conspicuous in the phase contrast photomicrographs (Fig 5)

Streptococcus faecalis

Pressing Str faecalis (Table 2, Fig 11) markedly reduces the number of colony forming bacteria The decrease in total count is not so conspicuous Estimation of the disintegration effect by the study of Gram-stained smears indicates an even lower degree of disruption In the counting chamber the untreated bacteria often occur in pairs, while pressed bacteria are almost always single

The extinction and turbidity of the material decrease with each successive treatment The soluble nitrogen is steadily increased by repeated pressings but the most viscous material is obtained after only one pressing As seen in the electron photomicrographs (Fig 12–16) of Str



Figs. 12–16.
Electron photomicrographs of Str. faecalis. (12) Nondisintegrated Str. faecalis. Bacteria pressed (13) once, (14) three times, (15) five times, (16) nine times. After five pressings dense bacteria are still found but they seem somewhat distorted. After nine pressings only smaller and larger cell wall fragments are recognized.

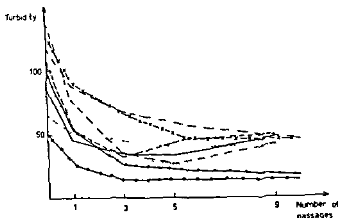


Fig 17

The turbidity after pressing different kinds of bacteria Turbidity values above 90 are obtained by extrapolation in Fig 20 from a lower dilution (—— E coli Proteus X 19 Lactobacillus bulgaricus – – – Clostridium perfringens x x x Bacillus megaterium – – – Staphylococcus aureus o-o-o- Streptococcus faecalis —● ●— Leuconostoc mesenteroides –– Mycobacterium phlei)



Fig 18 *Fig 19*

Fig 18

Supernatant from Lactobacillus bulgaricus pressed four times and centrifuged at 1 000 × g for 20 minutes The electron dense granules are not sedimented by centrifuging at 2 800 × g for 30 minutes but are sedimented by 12 000 × g for 30 min

Fig 19

Spores pressed three times Many spores are seen broken up











Figs 12–16

Electron photomicrographs of Str. faecalis. (12) Nondisintegrated Str. faecalis. Bacteria pressed: (13) once, (14) three times, (15) five times, (16) nine times. After five pressings dense bacteria are still found but they seem somewhat distorted. After nine pressings only smaller and larger cell wall fragments are recognized.

faecalis repeatedly pressed, the cell walls are fragmented into smaller units, but they are still recognizable after the ninth pressing This does not hold for E coli where the cell walls are disintegrated into very small units already after three pressings

To compare the sensitivity of different bacteria to disintegration, representatives of various families were treated in the small X-press The disintegration was estimated by turbidity (Fig 17) assay of the suspensions and by stained smears As seen in Table 3, the turbidity of all the bacterial suspensions investigated is reduced by the disintegration procedure, principally by the first few treatments The course of the reduction is however different from species to species The turbidity reduction usually corresponds rather well with the stained smears

When ... the family Lactobacteria- (Fig 18) They were seen ... eptococcus pyogenes and Lactobacillus bulgaricus and could be sedimented by centrifuging at 12 000 $\times$ g for 30 minutes but not by 2,800 $\times$ g for the same length of time

When B subtilis spores were pressed three times, the viable count dropped from 2 2 $\times 10^{8}$ bact/ml to 6 0 $\times 10^{7}$ bact/ml The electron photomicrographs (Fig 19) show that spore coats are fragmented

DISCUSSION

As found in earlier experiments (*Edebo* 1960) E coli are rather easily disintegrated The logarithms of the number of the total count and the viable count are almost linearly *decreased* with an increasing number of pressings, once the large reduction caused by the first pressing is excluded The significantly greater reduction by the first pressing might be due to the safety space, which results in some of the material not being pressed through the hole on each occasion In order to avoid damaging the disc of the X-press, the pistons cannot be pressed fully to the bottom of the compartment This leaves a space, the safety space, between the two of almost 1 mm and contains material which does not pass through the hole The once-treated material is collected ...
ty chamber ...
material pr...
tion is that ... in the first press ...

... survive better on ...

... the extinction and the turbidity as functions of the number of bacteria/ml give more linear than logarithmic

TABLE 3

Disintegration of different Kinds of Bacteria

The numbers show the turbidity In brackets are values extrapolated from Fig 20 The minus and plus show the disintegration estimated by stained smears

Bacteria	Number of pressings					
	No	1	3	5	9	10
Escherichia coli B	86	45 +++	34 ++++	33 +++++	46 +++++	
Proteus X 19	93 (106) −	53 ++	32 ++++	44 +++++	48 +++++	
Lactobacillus bulgaricus	93 (104)	60 +++	32 +++++	26 +++++		27 +++++
Clostridium perfringens	96 (138)	75 +	34 +++	26 ++++	40 +++++	
Bacillus megaterium	96 (127)	90 +++	66 ++++	46 ++++		44 +++++
Staphylococcus aureus 209	96 (118)	87 ++	67 +++	56 ++++		44 +++++
Streptococcus faecalis	91 (100)	54 ++	26 +++	22 +++		17 ++++
Leuconostoc mesenteroides	51	26 ++	14 +++	14 ++++		13 ++++
Mycobacterium phlei	65	50 +	45 +++	39 +++	40 +++++	

TABLE 4

total count extinction
tained corresponding to
these logarithmic values
pared

Number of passages	Total count log	Total count − log	Extinction 600 mμ Dilution 1 10	Extinction log	Extinction − log	Turbidity Dilution 1 10	Turbidity log	Turbidity − log
0	11 11		90 (1 100)	11 43		73 (1/100)	11.57	
1	10 17	94	96	10 46	97	76	10 62	95
2	9 89	.28	.82	10.38	08	64	10 45	17
3	8 97	92	.50	10 14	24	47	10 22	.23
5			.51	10 15	−01	48	10.23	−01
9			.54	10 18	−03	66	10 48	−.25

After the first pressing the logarithmic decrease of total count (0 94) is less than that calculated from the extinction and the turbidity (0 97 and 0 95 respectively) As light absorbing and scattering particles are formed as bacteria are disintegrated, the logarithmic reduction of total count should be greater than those calculated by photocell methods and thus the reduction of the total count by the first pressing is probably greater than calculated by the counting chamber In many cases it is very difficult to distinguish between disintegrated and non disintegrated bacteria The protoplasm probably continues to remain with the disintegrated cell Experience supporting this comes from Salton (1956) who found that cell walls from Gram negative bacteria were difficult to wash free from protoplasmic material In the pressings which follow the first reduction of total count exceeds those of the other methods, and this would be expected After a few pressings, the extinction of the bacterial suspension approaches an asymptotic value which might correspond to a suspension entirely made up of cell walls or the relatively insoluble cell wall fragments which appear upon disruption

Although Rayleigh's law for the scattering of light by particles in suspension is only true for dilute suspensions of particles which are small compared with the wave length, some qualitative information may be obtained by considering the different factors of the formula

$$\frac{I_S}{I_0} = \frac{n'^2 - n^2}{n^2} \cdot \frac{NV^2}{\lambda^4 r^2} (1 + \cos^2\beta)$$

(I_S = intensity of scattered light I_0 = intensity of incident light, n = refractive index of the medium n' = refractive index of the particles V = volume of each particle, N = number of particles, λ = wave length of incident light β = angle between the scattered and incident beams The particles are supposed to be contained in such a small

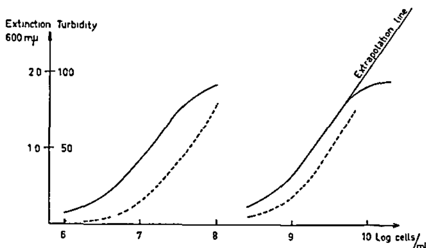


Fig 20

Relationship between logarithmic number (total count) of cells extinction and turbidity This graph is based on Saccharomyces cerevisiae (the two left curves) and a culture of E coli which is not identical with the one used in the disintegration experiments (the two right curves) (- - - extinction 600 mμ, —— turbidity)

plots However, significantly high concentrations were frequently enough employed to require the use of the logarithmic number of cells as more appropriate (Fig 20) When the extinctions and turbidities are plotted *vs* the total number of cells for different microorganisms, the slopes of the curves are equal but the position of the curves parallel-shifted along the abscissa but not equally so The relationship between the extinction and the turbidity of a suspension may thus be used to obtain data about the optical properties of the cell suspension It has been postulated that such properties are dependent on, among other *things, the size of the cells From Rayleigh's law (see below) it is known* that a certain mass made up of large particles scatters more light than if divided into smaller particles The observation (Fig 20) that, at the same extinction value, yeast cells scatter more light than E coli cells is thus expected

The linearly logarithmic relationship between the total counts and the extinction and turbidity values makes possible a conversion of the latter values into logarithms so that the logarithmic decrease (−Δ log, Table 4) of the total counts can be compared with the logarithmic decrease calculated from the extinction and turbidity values The total extinction and turbidity of the disintegrated suspensions are then estimated as *being derived from whole cells, although it is known that* some of the turbidity from the disintegrated samples is affected by subcellular particles, chiefly cell walls The curves for E coli of Fig 20 are thus used to obtain the logarithms corresponding to the various extinctions and turbidities This is possible because of the parallel shift of the curves which means that, when the differences between the logarithms are calculated, as done here, the error is corrected Table 4 shows the values for E coli obtained by the procedure just mentioned

When the viscosity was measured at $+20°$ C after centrifugation the supernatant from the material pressed once showed the highest viscosity This material was not thixotropic but the viscosity decreased with time probably due to the action of desoxyribonuclease(s)

Both the reduction of the viscosity and the loss of the anomalous behavior of the colloids may thus be due to an effect on the colloidal state of the macromolecules This seems very likely since DNA and RNA molecules are known to be fragmented when forced through a small orifice in the liquid state (*Cavalieri* 1957 *Littauer et al* 1960) and here the stresses imposed are probably much greater A secondary aggregation of the colloids may occur which involves both the cell walls and the cell wall fragments in the fashion seen under the microscope

When cell fragmentation occurs it may be explained by one or more of these possibilities (1) Shearing forces of the ice crystals of the flowing material (2) A sudden volume increase of the protoplasmic cell water at the phase shift from ice III to ice I (see below) or (3) The pressure gradients over the hole in the disk as suggested earlier (*Edebo* 1960) At the very beginning of the first pressing the bacterial protoplasm is better protected within the cells than once the cell walls are disrupted and protoplasm released There is a dramatic decrease in viscosity after pressing a second time This therefore suggests that the disintegrating effect is restricted either in space or time It follows that had the disintegration been a continuous rather than a sharply limited process th [illegible] een subjected [illegible] This did not [illegible] secondary to [illegible] sudden phase change of the ice rather than the shearing forces of the flowing ice The fact that disintegration occurs at temperatures above —22° C (*Edebo* 1960) when a change from ordinary ice (ice I) to liquid occurs further contradicts the first hypothesis

Electron photomicrographs indicate that the cell walls of *E coli* are disintegrated into very small units after repeated pressings It is believed that cell wall fragments have no interior water On the basis of this evidence it is quite improbable that at least as a single mechanism disintegration is caused by the sudden expansion of the cell water when ice III shifts to ice I (a 20 per cent increase in volume)

In certain respects the parameters of the disintegration of *Str faecalis* differ greatly from those of *E coli* The fact that the viable count of *Str faecalis* is much more reduced than the total count must be due to cell damage not visible in a light microscope at low magnification and not falsely low viable count due to aggregation of the cocci since no clumping of the cells is observed under the microscope Sufficient damage to the cell wall which does not allow escape of the cell content but removes the reproductive ability may have occurred but injuries other than these mechanical ones cannot be excluded

volume that the vectors r, *joining* the individual particles and the point of measurement, can be considered equal)

When the cells are disintegrated and their cell contents released, their refractive index, for which the cell walls may now be mainly responsible, is decreased (Fig 4) This is most likely the reason for the initial decrease in turbidity This decrease will be greater yet if the cell walls are disintegrated into smaller units The increase in turbidity after many pressings may be due to a precipitation of earlier soluble material or an agglutination of particles, the latter increasing the intensity of light scattering because this is proportional to the square of the individual particle volume but directly proportional to the number of particles

This view, based on Rayleigh's formula applied under rather nonideal *conditions, is supported by the other data Light- and electron micro* graphs show that both a disintegration of the cell walls into smaller units and an agglutination occur, and the decrease in both soluble nitrogen and viscosity supports the precipitation hypothesis The disintegration of the cell walls may be particularly useful as a means for making cell wall antigens accessible for gel-diffusion techniques

The greatest viscosity is found in samples which have only been pressed once Immediately after thawing, long, viscous filamentous aggregates can be seen with the naked eye in this material The material when undiluted, is also thixotropic, *i e*, it sets when standing in the ice water *bath Pouring, shaking and stirring result in a lowered viscosity* even at 0° C This is best demonstrated by streaming in the Ostwald viscosimeter There are progressively shorter flow times when a single sample is repeatedly streamed When the sample is allowed to stand the viscosity returns to a value slightly short of its original As expected the viscosity decreases again on streaming etc The decrease of viscosity usually involves a disorganization of weak structures by the stress applied, and the increased viscosity on standing is due to an orientation or association of the macromolecules in the form of clusters This anomalous behavior of the viscosity is particularly marked with linear *colloids of high molecular weight and is pronounced* at low temperatures (*Jirgensons* 1958) The viscosity did not return to its original value on standing in the Ostwald viscosimeter probably because of gel exhaustion or action of depolymerizing enzymes

When the bacterial material is pressed many times, the viscosity is decreased Since, at the same concentration, linear colloids are more viscous than spherocolloids and the viscosity (reduced viscosity extrapolater to zero *concentration) of linear colloids increases* with the molecular weight either a transition from a linear to a spheroidal state of the colloids, a decrease in molecular weight or both may have taken place here A reduction in the concentration of the colloids may also have occurred, but this is not likely when it is recalled that the nitrogen content of the supernatants does not parallel the viscosity

When the viscosity was measured at +20° C after centrifugation, the supernatant from the material pressed once showed the highest viscosity This material was not thixotropic, but the viscosity decreased with time, probably due to the action of desoxyribonuclease(s)

Both the reduction of the viscosity and the loss of the anomalous behavior of the colloids may thus be due to an effect on the colloidal state of the macromolecules This seems very likely since DNA and RNA molecules are known to be fragmented when forced through a small orifice in the liquid state (*Cavalieri* 1957, *Littauer et al* 1960) and here the stresses imposed are probably much greater A secondary aggregation of the colloids may occur which involves both the cell walls and the cell wall fragments in the fashion seen under the microscope

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TABLE 5

Disintegration of Str faecalis

Comparison of the disintegration of Str faecalis as measured by total count, extinction and turbidity By use of Table 20 logarithmic values are obtained corresponding to the extinction and turbidities The decrease (—Δ log) of these logarithmic values by the disintegration process are then compared

Number of passages	Total count		Extinction 600 mμ			Turbidity		
	log	—Δ log	Dilution 1 25	log	—Δ log	Dilution 1 10	log	—Δ log
0	11 06		1 38	11 12		47 (1/100)	11 22	
1	10 37	69	0 69	10 69	43	83	10 73	49
3	10 16	21	0 46	10 51	18	64	10 45	28
5	9 86	30	0 34	10 37	14	48	10 23	22
9	9 26	60	0 18	10 10	27	26	9 87	36

Use of Fig 20 to compare the total counts and extinctions and turbidities of Str faecalis yields the data in Table 5 This was obtained in a manner similar to that employed in compiling Table 4 After almost every pressing the logarithmic decrease of the total count exceeds that of the extinction and the turbidity This is expected since the light absorbing and scattering capacity is reduced but not abolished when a cell is disintegrated The fact that the turbidity is further reduced after each pressing, even when few cells are left to be disintegrated, may be recognized as a fragmentation of the cell walls into yet smaller units if Rayleigh's formula is qualitatively applied Electron photomicrographs support this hypothesis

The decrease in extinction and turbidity by the repeated pressing of Str faecalis proceeds much more slowly than with E coli and correlates with the greater resistance of streptococci to disintegration Also the total counts of Str faecalis fell more slowly than those of E coli

The viscosity of the streptococcal extracts is decreased by many pressings and a mechanism similar to that involved in E coli probably explains it However, the viscosity of the streptococcal extracts showed no anomalous behavior, probably because there are differences in the colloidal state of the protoplasms of E coli and Str faecalis

Electron photomicrographs show that the cell walls of Str faecalis are also disintegrated into smaller parts but contrary to the case with E coli, near-complete streptococcal cell walls may be easily found after the ninth pressing (Fig 16)

Great differences have already been shown between the susceptibility of E coli and Str faecalis to disintegration Other micro-organisms are differently susceptible too The curves obtained by plotting the turbidity vs the number of pressings vary from one micro organism to another Each such curve can be divided into two parts Part one consists of the first three pressings and mainly shows disintegration while part two groups the pressings which follow and reveals the tendency to

aggregation The dividing line between these two is not clearcut probably because bacteria, and mechanically resistant parts of them, are disintegrated with each successive pressing, and the effects on macromolecules are noted no later than the second pressing The reduction of the turbidity by disintegration is also dependent on the difference in light-scattering activity between the bacteria and their subcellular units

E coli and Proteus show the most rapid fall in turbidity, but later their turbidity increases E coli and Proteus belong to the group of bacteria which are most easy to disintegrate and like Clostridium perfringens, they have protoplasms which show a marked tendency to flocculate All other kinds of bacteria investigated show a more prolonged fall in their turbidity values The turbidity of Str faecalis and Staphylococcus aureus decreases continuously after each pressing which indicates that these bacteria and their cell walls are rather difficult to disintegrate Lactobacillus bulgaricus and Bacillus megaterium belong in an intermediate category with respect to their sensitivity to frag

tration curve show a strongly asymptotic tendency at nephelometric values below 20 The unique composition of the Mycobacterial cell wall probably also complicates the results Despite these factors, these micro organisms seem to belong to the group of bacteria difficult to disintegrate, since their curves are very shallow The difference in aggregation tendency after many pressings may be an expression of dissimilarities in the colloidal properties of the cell protoplasm of different species

The estimations of the resistance of bacteria to disintegration in the X-press deduced from the turbidity assays are generally supported by the stained smears and, in the case of E coli and Str faecalis, already shown to be true However, the Gram stained smears of pressed Cl perfringens show some irregularly stained rods They were counted as non disintegrated cells and possibly cause a slight underestimation of the disintegration Since the estimation of the degree of bacterial disintegration using stained smears yielded results falsely low for E col

... to disintegration

When Gram negative bacteria (E coli, Salmonella typhimurium, Bordetella pertussis) are pressed many times and observed under the electron microscope, usually only small cell wall fragments are recovered and no well shaped cell walls When Gram positive cocci (Str faecalis Str pyogenes Staphylococcus aureus) are treated in the same way however many well-shaped cell walls remain The resistance of Gram positive rods (Bacillus megaterium Lactob...

resistance of the different bacteria to disintegration by freeze-pressing in Hughes press (*Hughes* 1951) or in the X-press. This strongly suggests that the resistance of cells to freeze-pressing is dependent on the mechanical strength of their cell walls.

As a rule, the sensitivity of bacteria to disintegration in the X-press seems to be similar to that by ultrasonic treatment (*Grun & Steller* 1955). This suggests that the sensitivity to disintegration depends on some common factor, probably the mechanical strength of the cell wall, which has already been shown to be of great importance in the resistance to freeze-pressing.

Since Gram-negative bacteria are more susceptible to disintegration than Gram-positive cells this probably can be explained by the differences in cell wall composition. The cell walls of Gram-negative bacteria are more complex and contain the R-layer which is postulated to be responsible for the shape and mechanical strength of the cells (*Weidel Frank & Martin* 1960). This layer, roughly estimated to account for no more than 10 per cent of the dry weight of the complete wall of E. coli B, is the structure most similar to the cell wall of Gram-positive bacteria. Since the corresponding structures in most Gram-positive bacteria constitute a greater part of their cell walls, it is not astonishing to find a higher mechanical resistance. Gram-positive bacteria might well be expected to have a stronger cell wall than Gram-negative cells for another reason. The intracellular pressure of Gram positive bacteria (20–30 atmospheres) is supposed to be much greater than that of Gram-negative cells (5–6 atm.) (*Mitchell & Moyle* 1956).

The nature of the particles prepared from Lactobacillus bulgaricus is not yet investigated. They are unusually large compared with most subcellular particles from bacteria and they correspond, with respect to size and shape, mostly to polyphosphate granules (*Mudd, Yoshida & Koike* 1958).

The mechanism of disintegration in the X press is not completely elucidated. The disintegration of cell walls into smaller particles contradicts the hypothesis that disintegration is caused by the sudden volume increase of the protoplasm when the water changes from ice III to ice I, and the great decrease of the viscosity after the second pressing of E. coli plus the disintegration obtained at temperatures above —22° C contradict the postulation that the shearing forces in ice flowing under pressure are the basic causes of disintegration. It is more reasonable that the huge stresses and accelerations exerted on the bacteria particularly when they pass through the pressure gradient of the hole in the disk, seem to be responsible for a great deal of the disintegration effect. However, the passage through the hole cannot be entirely responsible for the disintegration because cells which have not passed through the hole are disintegrated to some extent. The potent effect obtained by *Hughes* (1951), when a pyrex glass abrasive was used for freeze-pressing, also stresses the importance of the shearing forces of

ice crystals The hypothesis that pressure gradients cause the disintegration of bacteria does have the advantage that it encompasses the other two hypotheses to some extent since the shearing and punching effect of sharp crystals on the bacterium may be considered as very sharply localized, microscopic pressure gradients, and the pressure difference between ice III and ice I is evident Macroscopic pressure gradients will cause an acceleration and flow of the material which makes a comparison with other disintegration methods fruitful The methods of *French et al* (1955) and *Ribi et al* (1959) which maintain the pressure gradients by the flow of liquid through a very narrow orifice, seem to be dependent on a very similar mechanism It has further been shown that the curve of the sonic (9kc) disintegration of E coli B can be imposed on the curve of the particle velocity in the center of the sonic cup, if suitable units are chosen, when the above parameters are plotted vs the plate voltage of the generator (*Kinsloe, Ackerman, Reid* 1954)

SUMMARY

A bacterial disintegration technique which depends on the pressing of bacteria in the frozen state (2,000 atm ,—25° C) through a 2.5 mm hole has been investigated in detail by treating Escherichia coli and Streptococcus faecalis and measuring the viable count, total count, extinction at 600 mμ, turbidity, viscosity and soluble nitrogen It is shown that the cell walls of the bacteria are disintegrated and the cell contents released When the material is pressed several times the disintegration of the cell walls progressively increases The viscosity of samples of disintegrated E coli is high and anomalous It is reversibly reduced by slight mechanical affection but irreversibly reduced by many pressings After repeated treatments of E coli, clumping occurs, and it is also observed with Proteus and Clostridium Samples of disintegrated Str faecalis are not so viscous as those of E coli and no clumping occurs after many pressings Similar results are obtained with Staphylococcus Lactobacillus and Bacillus species

The sensitivity of different bacteria to disintegration by this technique is similar to that found when other techniques are used The resistance of different bacteria to disintegration by freeze pressing seems to be dependent on the mechanical strength of the cell wall

The mechanism of the disintegration is discussed

REFERENCES

Bridgman P W Water in the Liquid and Five Solid Forms under Pressure Proc Am Acad Arts Sci 47 439 1912

Cat al .. [illegible] Acid and its Use in the Study of [illegible] 9 (4) 5319 1957

F leb [illegible] Micr Organisms and other Cells J [illegible] 188 4 451 1960

Edebo L & Cedergren B A Rapid Method for Preparation of Specimens for Electron Microscopy To be published
Edebo L & Hedén C G Disruption of Frozen Bacteria as a Consequence of Changes in the Crystal Structure of Ice J Biochem Microbiol Technol Engg 2 113 1960
French C S & Milner H W Disintegration of Bacteria and Small Particles by High Pressure Extrusion In Colowick and Kaplan Methods in Enzymology I p 64 Acad Press Inc Publishers New York 1955
Grün L & Stelter J Ultraschalleinwirkung auf Mikroorganismen Zeitschr f Hygiene *141* 267 1955
Hallberg V A New Method for Staining Tubercle Bacilli Applicable also to the Micro Organism of Leprosy and Other Acid Fast Germs Acta Med Scandinav Suppl 180 1946
Hallmann L Bakteriologie und Serologie 2 Aufl p 618 Georg Thieme Verlag Stuttgart 1955
Hughes D E A Press for Disruption of Bacteria and Other Micro Organisms Brit J Exp Path *32* 97 1951
Jirgensons B Organic Colloids Elsevier Publ Co 1958
Kinsloe Helen Ackerman Eugene & Reid J J Exposure of Microorganisms to Measured Sound Fields J Bact *68* 373 1954
Littauer U Z Danon D & Marikowsky Y Ribonucleic Acid from Escherichia coli Electron Microscopical Study Biochim Biophys Acta 42 435 1960
Mitchell P & Moyle Jennifer Osmotic Function and Structure in Bacteria Bacterial Anatomy Sixth Symp Soc Gen Microbiol 1956 p 150 Cambridge University Press
Mudd S Yoshida A & Koike M Polyphosphate as Accumulator of Phosphorus and Energy J Bact 75 224 1958
Ribi E Perrine F List E Brown W & Goode G Use of Pressure Cell to Prepare Cell Walls from Mycobacteria Proc Soc Exptl Biol Med *100* 647 1959
Salton M R J Bacterial Cell Walls Bacterial Anatomy Sixth Symp Soc Gen Microbiol 1956 p 81 Cambridge University Press
Tammann G Kristallisieren und Schmelzen pp 315 344 Barth Leipzig 1903
Weidel W Frank H & Martin H H The Rigid Layer of the Cell Wall of Escherichia coli Strain B J Gen Microbiol *22* 158 1960

FROM THE STATE BACTERIOLOGICAL LABORATORY, STOCKHOLM AND THE RHEUMATOLOGY DEPARTMENT, KAROLINSKA SJUKHUSET, STOCKHOLM

STUDIES ON THE EFFECT OF HOMOLOGOUS SPLEEN CELLS ON ANTIBODY FORMATION IN CORTISONE TREATED RATS

By

KAARE BERGLUND and ASTRID FAGRAEUS

Received 24 x 60

Previous investigations have elucidated some factors which condition the depressing effect of cortisone on antibody formation in the rat (*Berglund* 1956 a–d). In order to influence the early phase of the immune hemolysin response the hormone must be administered (by the intramuscular route) for at least 2 days before the antigen injection (*Berglund* 1956 a). Cortisone given after the day of antigen injection had no significant effect.

Further it has also been shown that this effect of cortisone can be inhibited by intraperitoneal injections of homologous spleen or thymus cells (*Berglund & Fagraeus* 1956). The present communication is a report on studies of some factors which condition this phenomenon: a) time of and number of spleen cell injections, and b) viability of the cells.

TECHNIC

Animals. White male rats weighing about 200 g were allotted at random to experimental groups (*Berglund* 1956). Blood samples of 1.0–1.5 ml were taken by heart puncture (experiment 1) or from tails (experiments 2 and 3).

Antigen. Fresh sheep erythrocytes were washed 3 times with saline and diluted with saline to the concentration used (*Berglund* 1956). The rats were immunized by a single intraperitoneal injection of 1 ml/100 g body weight of the antigen suspension.

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The cells were then spun down at 1 000 r p m for 10 minutes at 4° C They were resuspended in fresh Tyrode saline solution The volume was adjusted to contain a minimum of 10^8 nucleated cells/ml (10^8 cells have been obtained from 1/8 1/4 of a spleen in different experiments) *In experiments 1 and 2* the number of nucleated cells was determined in an ordinary counting chamber and another sample was viewed under phase contrast microscope Most cells did not appear damaged and after the addition of *trypan blue* very few showed any staining that would indicate death

A fresh cell suspension was prepared for every 24 hour period when cells were injected on more than one day The cells were kept at + 4° C until the injection

Hormone treatment Cortisone was given as daily intramuscular injections of a saline suspension of a commercial acetate (Cortal of Organon) in a dose of 4 mg per 100 g body weight per day Administration was started on day — 4 and finished with day + 1 In experiment 1 'controls' were given a corresponding amount of the agent used to suspend the cortisone acetate

Statistical treatment The Student's t test was used to compare the mean titers of two groups (*Fisher* 1948) Analysis of variance was performed according to directions given by *Snedecor* (1946)

EXPERIMENTAL

Experiment 1 Effect of Intraperitoneal Injection of Rat Spleen Cells —Given on Different Days—on Hemolysin Production in Cortisone Treated Rats (Tables 1 and 2)

Forty-eight rats weighing 175 210 g were allotted to six groups in numbers given in Table 1 Groups 1–5 were given cortisone as explained under "Technic" Group 6 was given the suspending agent of the cortisone acetate and served as control group All groups were immunized by a single intraperitoneal injection of a 0 4 per cent suspension of sheep erythrocytes

TABLE 1

Exp 1 *Production of Immune Hemolysin in Cortisone Treated Rats given Intraperitoneal Injections of Intact Homologous Spleen Cells on Different Days Antibody Titers Were Measured on Sera from Day + 5*

Group	Treatment	Rat spleen cells i p days	No of rats	Hemolysin titer M ± ε(M)
1	Cortisone		10	1 89 ± 0 33
2	Cortisone	0 + 1	7	4 40 ± 0 79
3	Cortisone	+ 1 + 2	7	1 49 ± 0 26
4	Cortisone	+ 2 + 3	7	2 53 ± 0 61
5	Cortisone	0→ + 3	7	2 37 ± 0 79
3–5	Cortisone	+*	21	2 13 ± 0 34
6	Suspending agent Controls		10	4 34 ± 0 62

* See the component groups of this pooled group

Groups 2 5 were given intraperitoneal injections of spleen cells as follows *Group 2* On day 0 one injection 5 hours after the antigen injection and another 6 hours later (each containing approximately 2 10^8 cells) On day + 1 two injections at a 12 hour interval (each approximately 3 10^8 cells) *Group 3* Four injections 12 hours apart

on days + 1 and + 2 (each approximately 3 10⁸ cells) *Group 4* Four injections 12 hours apart on days + 2 and + 3 (each approximately 3 10⁸ cells) *Group 5* Two injections every day from day 0 through day + 3 simultaneous with the spleen cells injections of other groups and containing the same amount of cells as these

The animals were bled on day + 5 The group means of hemolysin titers are given in Table 1

TABLE 2

Exp 1 Differences (D) in Mean Hemolysin Titer between the Groups of Table 1

Groups compared	Hemolysin titer D ± s(D)
1 and 2	2 51 ± 0 77 *
1 and (3 5)	0 24 ± 0 55
1 and 6	2 45 ± 0 70 *
2 and (3 5)	2 27 ± 0 75 *
2 and 5	2 03 ± 1 12

Results As expected animals receiving cortisone but no spleen cells produced *significantly* less hemolysin than those of the control group (comparison of groups 1 and 6 Table 2) The four groups given cortisone *and* spleen cells differed considerably in mean hemolysin titers A one sided analysis of variance applied to these groups showed that all groups *probably* (P<0 05) did not belong to the same population This *heterogeneity could be eliminated by omitting group 2* which had *significantly* higher titers than the other 3 groups (Table 2) The remaining groups (groups 3 to 5) were pooled (Table 1) and compared with group 1

Administration of *spleen cells on day 0 and day + 1 significantly* increased hemolysin titers in cortisone treated animals (comparison of groups 1 and 2 Table 2) Injections of spleen cells on other days did not significantly alter antibody levels on day + 5 (comparison of group 1 and group 3 5 Table 2) In fact animals *given cells on days 0* and + 1 had *significantly* higher titers than those injected on other days (comparison of group 2 and group 3 5 Table 2) The last compar... in

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... A direct comparison of the relevant groups (2 and 5) did not however give statistical support for such a statement (Table 2)

Experiment 2 Effect of Intraperitoneal Injections of Rat Spleen Cells —Variation of the Number of Injections from 1 to 4 (Table 3)

Twenty three rats weighing *190 210 g* were allotted to four groups in numbers given in Table 3 All groups were given cortisone as ex

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plained under "technic". The animals were immunized by a single intraperitoneal injection of a 0.1 *per cent* suspension of sheep erythrocytes.

Spleen cells were given intraperitoneally to groups 2–4 as follows. Group 2 received four injections—the first 2½ hours, the second about 8 hours, the third about 18 hours, and the fourth about 27 hours after the antigen injection. Group 3 was given two injections on day 0, equal in all respects to the two first given to group 2. Group 4 received only one injection, equal to the first given to groups 2 and 3. Each injection had a volume of 1 ml. The injections, given during the first 24 hour period after the antigen injection, each contained about 2.6×10^8 cells. No cell count was performed with the cell suspension prepared for the last injection in group 2.

The animals were bled on day + 5. The hemolysin titration was performed with 4-fold dilution steps. By a mistake titration with 2 fold dilutions could not be done later. However, the titration error–in four-fold titrations—is nevertheless small in comparison to the individual variation within groups (*Berglund* 1956), and does not jeopardize the conclusions. The group means of hemolysin titers are found in Table 3.

Statistical analysis. An one-sided analysis of variance, applied to all groups, indicated heterogeneity between groups ($P < 0.05$). When group 1, that had not received spleen cells, was omitted, analysis of variance showed no significant heterogeneity between the spleen cell treated groups (2–4). These groups could therefore be pooled into one group (Table 3).

TABLE 3

Exp 2. Production of Immune Hemolysin in Cortisone Treated Rats Given one (Day 0) two (Day 0) or four (Days 0 and + 1) Intraperitoneal Injections of Intact Homologous Spleen Cells. Antibody Titers Were Measured on Sera from Day + 5

Group	Treatment	Rat spleen cells i. p. no of inject	No. of rats	Hemolysin titer M ± ε(M)
1	Cortisone		8	1.98 ± 0.35
2	Cortisone	4	5	3.14 ± 1.00
3	Cortisone	2	5	3.20 ± 0.93
4	Cortisone	1	5	4.80 ± 0.30

Pooled group (2–4). Mean hemolysin titer = 3.71 ± 0.48
Difference between group 1 and group (2–4) = 1.73 ± 0.71*

Results. Four injections of spleen cells, given between 2½ to 27 hours after the antigen injection (a. i.), or two such injections, given 2½ and 8 hours after a. i., did not lead to hemolysin responses in cortisone treated rats, that significantly differed from those found in animals receiving only one such injection at 2½ hours after a. i. The spleen cell treated animals, considered as one group, produced *probably* higher hemolysin titers than animals given cortisone only ($P < 0.05$).

Experiment 1 showed that four injections of spleen cells could increase the hemolysin production in cortisone treated rats if they were given on day 0 and day + 1 In experiment 2 it was found that one injection of spleen cells on day 0 was as effective as 1) two such injections on day 0 and 2) four distributed on day 0 and day + 1 In the following experiment spleen cells were—with one exception—accordingly given as single injections on day 0

Experiment 3 Effect of Intraperitoneal Injection of Damaged Spleen Cells on Hemolysin Production in Cortisone Treated Rats
(Tables 4 and 5)

Forty five rats were allotted to 6 groups in numbers given in Table 4[1] Groups 1–4 were given cortisone as outlined under technic Groups 5 and 6 received no cortisone All groups were immunized by a single intraperitoneal injection of a 0 1 per cent suspension of sheep erythrocytes

Groups 2 5 were given intraperitoneal injections of cells taken from a common pool of spleen cells

Groups 2 and 5 were given a single injection of intact spleen cells 2 hours after the antigen injection (day 0) The cell suspension was injected in a volume of 3 ml per animal Each rat received the average number of cells obtained from 3 8 of a spleen (see technic) No cell count was performed In several earlier experiments the average number of cells obtained from one spleen varied between 4 8 10^8 Accordingly it could be estimated that these animals received 1 3 10^8 cells each

Group 3 received cells which had been killed by incubation at 50° C for 1 hour Otherwise the injection was in all respects identical to the [illegible] this suspension revealed no intact cells All animals received four injections Each injection had a volume of 1 5 ml equivalent to half the amount of cells given to groups 2 3 and 5 Accordingly the rats of group 4 received a total amount of cell fragments obtained from double the number of cells given to the other groups The first injection was given 2 hours after the antigen injection and the three following at 2 hour intervals This spreading of injections was instituted as a possible compensation for a more protracted effect of viable cells

The animals were bled on day + 5 and day + 8 As the titers on day + 5 were too low to permit calculations only titers from day + 8 will be reported (Table 4)

Results Administration of intact normal spleen cells did not significantly alter hemolysin response in animals not treated with cortisone

[1] One animal was lost before day + 8 in each of the groups 2 3 and 4

(comparison of groups 5 and 6, Table 4). The two groups, which did not receive cortisone, could thus be pooled into one control group (group 5 + 6, Table 4).

TABLE 4

Exp 3 *Hemolysin Production in Cortisone Treated Rats Given Intraperitoneal Injections of Damaged Homologous Spleen Cells When not Otherwise Indicated Cells Were Given as a Single Injection on Day 0 Antibody Titers Were Measured on Sera from Day + 8*

Group	Treatment	Kind of cells given i p	No of rats	Hemolysin titer M ± ε(M)
1	Cortisone	—	10	0 41 ± 0 28
2	Cortisone	Intact spleen cells	9	2 69 ± 0 71
3	*Cortisone*	*Heat treated spleen cells*	4	*0 23 ± 0 10*
4	Cortisone	Disintegrated spleen cells —4 injections	9	0 22 ± 0 08
5	No	Intact spleen cells	5	3 04 ± 0 89
6	No	—	5	2 58 ± 0 62
5+6	No "Controls"	(see groups 5 and 6)	10	2 81 ± 0 52

TABLE 5

Exp 3 *Differences (D) in Mean Hemolysin Titer between the Groups of Table 4*

Groups compared	Hemolysin titer D ± ε(D)
1 and 2	2 28 ± 0 73**
1 and (5+6)	2 40 ± 0 59***
2 and 3	2 46 ± 1 10*
2 and 4	2 47 ± 0 72**

Cortisone *significantly* reduced antibody titers in animals which were not given cells (comparison of group 1 and group 5 + 6, Table 5) A single injection of intact normal spleen cells *significantly* increased the hemolysin response in cortisone treated animals (comparison of group 1 and group 2, Table 5) Killing of cells 1) by heat *probably* and 2) by disintegration *significantly* reduced this effect of spleen cells (comparisons of group 2 with groups 3 and 4 respectively, Table 5) The mean hemolysin titers of groups given cortisone and damaged cells showed no significant difference from that of the group given cortisone alone (groups 1, 3 and 4, Table 4)

COMMENTS

Previous experiments have indicated that the inhibiting effect of cortisone on the immune hemolysin response in the rat is due to an interference with an early part of the antibody production process Administration of cortisone from the day following the day of antigen injection (day + 1) did not influence the early phase of the antibody response (*Berglund* 1956 a) Accordingly, it was of considerable interest to investigate whether a similar time relation characterized the restora-

tion of the antibody formation by homologous spleen cells in cortisone treated rats

The result of experiment 1 indicates that normal intact spleen cells—in the dosage used—have little or no effect when the administration is started on day + 1 or later From experiments 2 and 3 it is apparant that a single intraperitoneal injection of spleen cells 2 hours after the antigen injection (day 0) suffices to neutralize the effect of cortisone on antibody formation In fact, additional injections on day 0 and day + 1 could not be shown to increase this effect Further injections on day + 2 and day + 3 seemed to decrease rather than increase the effect of cells given on day 0 and day + 1 (experiment 1)

These findings may be summarized as follows As cortisone must be administered before day + 1 to suppress the early phase of the immune hemolysin response, homologous spleen cells have to be given before day + 1 to neutralize this effect In a previously published experiment (*Berglund & Fagraeus* 1956) spleen cells given 22 hours before the antigen injection (day — 1) had no effect Other points of time for the spleen cell transfer—between 22 hours before and 2 hours after the antigen injection—have not been investigated

The mechanism by which cortisone interferes with the antibody formation process is not known (*Berglund* 1956 a) The present experience suggests that the injury is located very early in this process—probably deranging reactions that take place during the first 24 hours after the antigen injection Phagocytosis is the only well known phenomenon in this phase Studies of the effect of cortisone on ingestion of antigen in phagocytes have given conflicting results (reviewed by *Kass & Finland* 1953 and *Timiras* 1953) On the other hand, investigations on digestion of antigen by phagocytes during cortisone administration have unanimously shown a retardation of this process (*Lurie et al* 1952, *Kass et al* 1953 and *Clawson & Nerenberg* 1953) This observation could explain a delayed antibody response but not the permanent inhibition observed (*Berglund* 1956 c)

The observation that the cortisone-neutralizing effect of intact normal spleen cells is also limited to the day of antigen injection suggests that the transferred cells may correct a specific derangement caused by cortisone They may take over a cellular function in the assimilation of antigen or may produce a humoral factor that restores the recipient's own cellular function in this respect If the transferred cells take an active part in the antibody forming process, do they also synthesize the excess of antibody produced in these animals compared to those given cortisone only? Th
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to rats (*Fagraei*
transferred cells ... demonstrated

However, a non-specific stimulation of antibody production by the transferred cells can not be disregarded The intraperitoneal injection

(*comparison of groups 5 and 6, Table 4*). The two groups, which did not receive cortisone, could thus be pooled into one control group (group 5 + 6, Table 4)

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Berglund K & Fagraeus A A biologic factor inhibiting the effect of cortisone on antibody production Nature *177* 233 1956

Clawson B J & Nerenberg S T The effect of large doses of cortisone upon the ability of the reticuloendothelial cells to phagocytose streptococci J Lab Clin Med *42* 746 1953

Fagraeus A & Berglund K Inhibition of the effect of cortisone on antibody formation by heterologous spleen cells J Immunol *87* 49 1961

Fisher R A Statistical methods for research workers Hafner New York 1948

Kass E H & Finland M Adrenocortical hormones in infection and immunity Ann Rev Microbiol *7* 361 1953

Kass E H Kendrick M J & Finland M Effect of certain corticosteroids and of growth hormone on nucleoproteins of lymph nodes Ann New York Acad Sc *56* 737 1953

Lurie M B Abramson S Heppleston A G & Dannenberg A M Immunological and hormonal studies on the nature of genetic resistance to tuberculosis

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of homologous cells means introduction of considerable amounts of biological material—probably partly antigenic This may give rise to an adjuvant effect In experiment 2 heatkilled, but morphologically intact cells, did not significantly counteract the effect of cortisone on antibody formation Further, disintegration of cells—thereby reducing denaturation of the biological material, but losing the morphological form of the cell—had no demonstrable effect These results, as well as similar experiences with heterologous cells (*Fagraeus & Berglund* 1961), do not support the hypothesis that restoration of antibody formation by spleen cells in cortisone treated rats is due to an adjuvant type of mechanism

SUMMARY

The investigation is a study of some factors that condition the effect of homologous spleen cells on antibody formation in cortisone treated rats The test object has been the hemolysin response in rats immunized by a single intraperitoneal injection of sheep erythrocytes and given cortisone by a schedule leading to depression of antibody formation

A single intraperitoneal injection of intact normal rat spleen cells —given on the day of antigen injection—sufficed to give a significant increase of hemolysin titers in cortisone treated rats Four injections of such spleen cells—two on the day of antigen injection and two on the following day—were also effective When additional cell injections were given on the two following days (day + 2 and day + 3) no significant effect was obtained—indicating a possible negative effect of cell administration on these days Further, injections of intact normal spleen cells started on days following the day of antigen injection did not restore hemolysin formation in cortisone treated rats

Spleen cells—killed by heat or disintegration—did not increase antibody formation in animals treated with cortisone This observation makes an adjuvant effect of intact cells less probable

It is suggested that—in the experimental model used—cortisone interferes with a phase of the antibody production process limited to the first 24 hours after the antigen injection The effect of living, homologous spleen cells—when administered during this period—suggests that this cortisone-induced derangement is thereby corrected

REFERENCES

Berglund K Hemolysin response in the rat after a single intraperitoneal injection of sheep erythrocytes Acta path et microbiol scandinav *38* 220 1956

Berglund K Studies on factors which condition the effect of cortisone on antibody production

1 The significance of time of hormone administration in primary hemolysin response Acta path et microbiol scandinav *38* 311 1956 a
2 The significance of the dose of antigen in primary hemolysin response Ibid *38* 329, 1956 b
3 The significance of time of hormone administration in primary agglutinin response to *S typhi H* Ibid *38* 403 1956 c
4 The significance of the particle size of the antigen Ibid *39* 41 1956 d

komm 1957a and reviewed more in detail by *Lucke & Schlumberger* 1949, who find no conclusive evidence on real formation of tumours, but emphasize that experimental conditions have in most cases been inadequate In 1951, however, *Breedis* reported the induction of transplantable spindle cell sarcomas in Triturus viridescens More than 500 newts injected in the forelimbs with various carcinogens had in 11 per cent to 40 per cent of cases developed accessory limbs, duplicating the injected leg In two cases only, malignant tumours developed following the injection of 3 methylcholanthrene, 1 mg in olive oil Lantency periods were 168 days and one year respectively

Leone 1958, implanted pure crystals of methylcholanthrene subcutaneously in 47 newts, Triton cristatus, and observed a local neoplastic infiltration in about 40 per cent of the animals, developing in the course of a few months and accompanied by distant metastases Transplantation showed a high percentage of takes

The Basis of the Newt Test

In their attempts at producing tumours in salamanders by chemical means *Hellmich* 1929, *Koch, Schreiber & Schreiber* 1939, and *Pflugfelder* 1949, observed an early proliferation of the epidermis, with no or only slight atypical features and with some tendency to local invasion Similar lesions in axolotls were reported by *Fedatov* 1941 and by *Finkelstein* 1944 The epithelium seemed normally differentiated and the lesion was probably not neoplastic *Lucke & Schlumberger* 1949, regard this lesion as "granulomatous masses or as non neoplastic hyperplasia resulting from prolonged irritation" which, however, would not seem to exclude its applicability as a criterion for carcinogenicity

This application of the skin reaction has been made by *Neukomm* 1957, 1959, who originally studied the effect of tar, benzpyrene, and methylcholanthrene on the skin of newts in neutral regions, and in regions particularly capable of regeneration such as the tail, 1944 Both kinds of location showed an epithelial reaction about [illegible]

[illegible] widened, affecting not only the subcutaneous tissue including its glands but also the superficial muscular layer On the surface an ulceration developed, but the proliferating epithelium showed no sign of atypica cells and their nuclei looked normal, and mitoses were rare From about the 40th day the epithelial infiltration regressed, and was delimited by a reaction of connective tissue after which resorption nearly restored normal conditions

Neukomm found tar, benzpyrene, and methylcholanthrene 100 per cent effective in producing this reaction, when administered repeatedly as 0 25 per cent to 5 per cent solution in pure olive oil Variation in

FROM THE DEPARTMENT OF PATHOLOGY, THE FINSEN INSTITUTE, AND THE DANISH CANCER REGISTRY DIRECTOR JOHANNES CLEMMESEN, M D

STUDIES ON THE NEWT TEST FOR CARCINOGENICITY

1 *Benzo[a]pyrene, Dibenz[a,h]anthracene and 3-Methylcholanthrene*

By

E ARFFMANN and B COLLATZ CHRISTENSEN

Received 20 xii 60

In a recent review one of us (A) has demonstrated the insufficiency of our present tests for carcinogenicity The ever increasing environmental exposure to new chemical compounds is increasing the need for such tests, and the development of quicker tests than the usual would seem particularly valuable Although it may be expected that biological tests for carcinogenicity will to some extent be unspecific, it may on the other hand be hoped that, like the case is in bacteriology, combinations of such imperfectly specific tests will give important information

It is on this background that the authors have studied the applicability of the socalled newt test previously applied by *Neukomm* 1957

Cold-Blooded Vertebrates in Experimental Carcinogenesis

According to *Schlumberger & Lucke* 1948, tumours in cold-blooded animals will largely show the same structure and behaviour as corresponding tumours in warm blooded animals Spontaneous tumours may not be quite as rare as previously assumed, and are rather often suited for experimental studies, as reported by *Lucke & Schlumberger* 1949 In newts carcinomas have been demonstrated in the testes, skin glands and the skin, and fibromas in the subcutaneous tissue In the axolotl, hereditary melanomas are wellknown, and so are virus-induced renal adenocarcinomas in the frog, Rana pipiens

Amphibia were used by *Champy & Vasiliu* 1924 (*Champy & Champy* 1935), and by *Hellmich* 1929, in experimental carcinogenesis, largely with a view to their ability to local growth and regeneration Further advantages of cold blooded animals to the study of tumours are their slower speed of biological processes, and their dependance on the temperature of the surroundings combined with a longer period of survival *in vitro* of their tumour tissue, according to *Lucke & Schlumberger* 1949

Earlier experiments on chemical carcinogenesis in amphibia, particularly newts and axolotls have been surveyed schematically by *Neu-*

size and covered by a metal net Thirty to fifty animals were kept together and fed weekly on finely hashed liver *More frequent meals may cause deaths due to over* eating Other dangers are pollution of the water by food remnants or faeces, and fungus infection by Saprolenia which may be prevented by euflavin in weak solution In this way the newts may be kept ready for experiments at all seasons but breeding in captivity is considered extremely difficult

TABLE 1

Experiments on Secondary Tails

Experiment No	IX			X	
Substance injected	BP* in peanut oil	Soya bean oil	Peanut oil	BP* in peanut oil	Peanut oil
Number of animals	9	10	9	9	9
Epidermal reaction on					
7 day after injection	—	—	±	+	+
10	—	—	—	—	+
15	—	+—	—	+	—
20	—	—	—	+	—
30	—	—	—	(+)	—
40	—	—	—	—	±
50	—	—	—	+	—
60	—	—	—	+	—
70	lost	—	—		
Animals died during experiment	0	0	0	1	1

* BP = benzo[a]pyrene

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was about 7 per cent Out of 21 casualties 7 animals were usable for h... ...ortality among experimental animals examination and were ...

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number of injections did not significantly alter the latency period In control experiments with *pure olive oil only slight connective tissue* reactions appeared, and the specificity of the test was further examined in connection with studies on the carcinogenic qualities of fractions of cigarette tar, 1957 b Triton cristatus received one subcutaneous injection into the tail of a 0 1 per cent solution or suspension in olive oil (*Neukomm* 1957 a) Injections with benzpyrene, dibenzanthracene, and methylcholanthrene produced typical positive reactions showing epithelial hyperplasia with infiltrating downgrowth about the 10th and 18th day Quantitative studies showed positive reactions on 10^{5} to 10^{9} g/l of the three carcinogens mentioned It seems particularly interesting *that a number of non-carcinogenic hydrocarbons, although of relatively* simple constitution, such as benzene, phenol, naphtalene, acenaphtene, anthracene and pyrene elicited no response, so that studies of non-carcinogenic compounds of a more complicated structure closer to the carcinogens would seem indicated Chrysene and 1 2 benzanthracene which compounds are weakly carcinogenic to mammals were active on injection into Triton cristatus

The correspondence between *Neukomm's* results in newts and the reported results in mammals was less clear for nitrogenated hydrocarbons and azo dyestuffs, and he restricted his statement to the conclusion that the newt test might replace the usual tests on mammals as far as polycyclic hydrocarbons are concerned

In 1959 he reported in more detail on the histological criteria for the test, *emphasizing* that a combination of hyperplasia and downgrowth (through the pigmented layer between the epithelium and the dermis) is prerequisite to counting a result as positive By comparison with a quick test with the degeneration of sebaceous glands in mouse skin as indicator he found that with a few exceptions compounds showing a sebaceous gland suppression index exceeding 300 will also give positive results in newts

It would, however, seem to the present authors that a semiquantitative use of the newt test, as practised by *Neukomm & Luder-Huguenin* 1960, will be open to some criticism After all, the grading of the test is based on a fairly subjective evaluation of the histological response, as illustrated later in this paper, and the quantitative estimate is worked out on the basis of groups of six newts each, while control animals are not mentioned In these studies, fluorene, 2 aminofluorene and 2 acetylaminofluorene, gave negative results when *tested*

Nevertheless, whether or not the newt test is fully specific for carcinogenicity in mammals, it seems that its background is worthy of further study

TECHNIQUE

In our studies we have used Triton cristatus imported partly from the Po valley partly from the *Low Countries* The animals have been kept in running water originally in glass aquaria containing 5 to 6 l later in plastic vessels of corresponding


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HISTOLOGICAL TECHNIQUE

With the injection technique described an amputation of the tail near the anus will provide for histological study a piece stretching from the puncture marked by the suture, and 0.5–1 cm in oral direction.

Following Neukomm's technique we have fixed the tissue for 8 days in 4 per cent neutral formaline. Calcium is extracted from the bone by storage in 5 per cent trichloracetic acid for three days, after which the tissue is kept for six hours in physiological saline, followed by 4 per cent formaline for 12–18 hours. Embedding in paraffine follows, according to the usual procedure.

In order to hit the often minute lesion at the point of reaction each tissue block has been cut into several pieces embedded together, and later cut in serial sections of 5 μ, of which every fourth was used for staining. In most cases the entire block was cut, leaving 50 to 100 suited sections from each tail. These were stained by hematoxylin eosin and usually histologically examined by at least two investigators.

HISTOLOGICAL RESULTS

The surface of the tail of the newt is covered by squamous epithelium with 3 to 5 layers of cells (Fig. 1). Immediately beneath the basal membrane is a clear layer of pigmented cells. The major part of the dermis is occupied by densely situated, roundish glands opening on the surface. Centrally in the tail the spine surrounds the medulla with a big artery and vein on the anterior side. The spine is surrounded by large muscles.

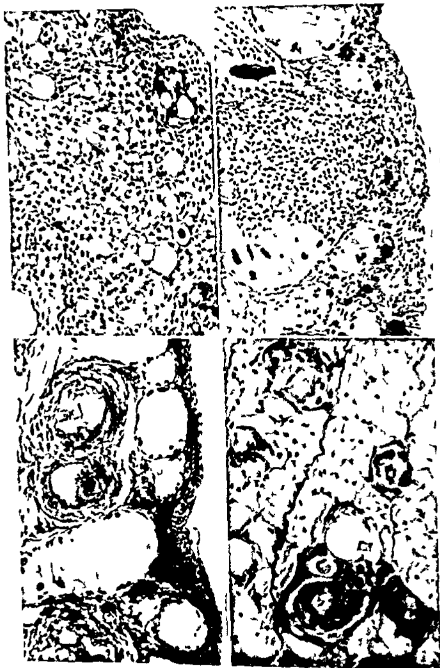
The histological evaluation of the newt test is usually quite simple, but may in some cases raise a few questions. The estimate of epithelial hyperplasia should, for instance, consider that some variation in the thickness of the epithelium occurs normally. It may even be apparent only, and caused by asymmetrical embedding of the tail pieces, so that a slight diffuse "hyperplasia" limited to one side should not be counted. Contrarily a vigorous response, for instance to the injection of dibenzanthracene, may reduce the epidermis through partial ulceration.

In the main we have applied the criteria given by Neukomm 1957 etc. who emphasized that a reaction grouped as positive must show hyperplasia combined with downgrowth of epithelium. We have, however, found it logical to designate a clearly local hyperplasia as partly positive, (+), because such epithelial proliferation must represent the first stage in the response of the tissue. On the other hand we have not attempted any quantitative gradation of positive results. Doubtful reactions have been represented by ±.

Even in clearly negative cases it is easy to find the site of the injection by means of the oil droplets, which persist for many weeks.

Figs. 1–4

Fig. 1. Normal tail of the newt. To the upper right epidermis below the musculature.—*Fig. 2.* Hyperplasia of epithelium on 7th day after methylcholanthrene injection. No penetration of pigmented layer. (+). *Fig. 3.* Hyperplasia of epithelium on 15th day. penetration of pigmented layer. + — ... hal islet close to oil droplet on 9th day.


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8

In a few cases tissue reaction to a carcinogen was observed already on the third day, but generally the epithelial proliferation does not seem to develop until the end of the first week In positive cases local hyperplasia had usually developed at this time (Fig 2), and in some animals a single or a few epithelial cords (Fig 3) could be seen pushing down between the subcutaneous glands, so that a single section would show an epithelial islet in the dermis (Fig 4) At this time it is not common to find extensive epithelial proliferation, which usually culminates between the 12th and 21st day, in typical cases with extensive and irregular epithelial downgrowth into the dermis, accompanied by destruction of glands and invasion of the superficial layers of the musculature (Fig 5) Like *Neukomm* we have always found the proliferations consisting of well differentiated epithelium

Regarded with the naked eye the surface of the area injected is either prominent on account of hyperplasia, or slightly depressed due to partial ulceration At some time between the 20th and the 30th day the epithelial reaction begins to regress, and is no more discernible from the 40th to 50th day

Unspecific reactions as inflammation, and, later, connective tissue formation, is observed to a varying extent During the first days an acute infiltration by leukocytes may develop, taking a more chronic course within one or two weeks (Fig 6) Twenty to thirty days after injection lipophages may appear around the oil droplets, but from forty to fifty days after injection a concentric formation of connective tissue forms a kind of capsule around them (Fig 7) Necrotic cells may occur within the droplets at this time

This mensenchymal reaction is also seen following injections of the pure oily solvents *At histological examination following a pronounced* connective tissue response (Fig 8) it is usually impossible to distinguish between the remnants of an epithelial reaction immediately surrounding the oil droplets, and connective tissue with lipophages Staining *according to van Gieson-Hansen is of no avail*

In accordance with *Neukomm* we have found the optimal period for the estimate of the tissue reaction to cover the second and third week following injection

GENERAL RESULTS

As it will appear from Table 2, our first experiments have been separated from the others, because a positive reaction to the solvent was observed in nearly half the controls Contamination with carcinogen

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Figs 5 8

Fig 5 Strong hyperplasia of epithelium with broad penetration of pigmented layer on 30th day after methylcholanthrene injection + *Fig 6* Unspecific inflammatory reaction on *peanut oil 40th day* *Fig 7 Proliferation of connective tissue* around oil droplets on 70th day after peanut oil injection *Fig 8* Isolated proliferation of cells around oil droplets in muscle on 60th day after peanut oil injection

TABLE 3 a

Experiments with benzo[a]pyrene *in various solvents*

Experiment No	XII		XIII		XIII		XIV		XV		XVI	
Substance	BP* in peanut oil	Peanut oil	BP in peanut oil	Peanut oil	BP in pea nut oil	Peanut oil	BP in soya bean oil	Soya bean oil	BP in olive oil	Olive oil	BP in peanut oil	Peanut oil
Number of animals (Triton cristatus)	9	9	15	9	7	7	7	7	7	14	7	7
Epidermal reaction on												
3 day					±	—	—	—	+	see under MCA	—	see under MCA
6 – 7 –	—	—	++	±	—	—	+	—	+		+	
9 – 10 –	+	—	—+	—	—	—	+	—	+			
12 –					+	—	+	—	(+)		(+)	
15 –	+	—	—+	—	—	—	—§	—	—		(+)	
18 –					—	—	—	—	—		—	
20 21 –	—±	—	—(+)	—	—	—	—	—	(+)			
30 –	+	—	(+)—	—								
40	+	—	+—									
50 –	—	—	—									
60 –		—	—									
70 –		—										
Animals died during experiment	1	0	1	4	0	0	0	0	0	0	2	1 (included)

* BP — benzo[a]pyrene
§ No signs of the injected oil

TABLE 2

Discarded Experiments

Experiment No	A		B		C	
Substance injected	MCA* in peanut oil	Peanut oil	DBA* in peanut oil	Peanut oil	BP* in peanut oil	Peanut oil
Number of animals	9	6	9	6	6	5
Epidermal reaction on 4 day after injection						
9–10	— —(+)	—+	+++	(+)	—/+	+—
13–15	+§	+—	+++	+—	++	(+)—
19–21	+++	— —	+++	—†	+	+
Animal died during experiment	0	0	0	1	2 (one included)	0

* MCA = 3 methylcholanthrene DBA = dibenz[*a h*]anthracene BP = benzo[*a*]pyrene

§ Two showed no cysts at the injection site

† One showed no cysts at the injection site

can be excluded, except perhaps in the first experiment, in which injections of carcinogen took place earlier than the control injections Whether the response to the solvent may be due to decomposition of fats will be subject to further study

In Table 3 (a, b, c) we have presented the results of injection experiments on 138 newts, with the potent carcinogens 3-methylcholanthrene, dibenz[*a h*]anthracene and benzo[*a*]pyrene Control injections of the pure solvents were given at the time to a total of 128 newts In some cases more than one carcinogen were tested together

CONCLUSION AND SUMMARY

The newt test for carcinogenicity applied by *Neukomm* has been repeated in accordance with his technique

The authors have confirmed that proliferation of the epithelium regularly follows the injection of benzo[*a*]pyrene, dibenz[*a,h*]anthracene and 3 methylcholanthrene solved in peanut oil and in a few experiments in soyabean oil, or according to *Neukomm*'s technique, olive oil

Dibenzanthracene was found the most potent compound, yielding the highest incidence of epithelial reaction in the newts

The secondary tail does not seem to respond to the carcinogens with the same regularity as the primary

The test as a whole may be carried out within few weeks, but its demands with regard to histological examination are fairly heavy Another limitation is the seasonal character of the availability of test animals

TABLE 3 a

Experiments with benzo[a]pyrene *in various solvents*

Experiment No	VII		VIII		XIII		XIV		XV		XVI	
Substance	BP* in peanut oil	Peanut oil	BP in peanut oil	Peanut oil	BP in pea nut oil	Peanut oil	BP in soya bean oil	Soya bean oil	BP in olive oil	Olive oil	BP in peanut oil	Peanut oil
Number of animals (Triton cristatus)	9	9	15	9	7	7	7	7	7	14	7	7
Epidermal reaction on												
3 day					±	—	—	—	+	see under MCA	—	see under MCA
6 – 7	—	—	++	±	—	—	+	—	+		+	
9 –10	+	—	—+	—	—	—	+	—	+			
12					+	—	+	—	(+)		(+)	
15	+	—	—+	—	—	—	—§	—	—		(+)	
18					—	—	—	—	—		—	
20 21	—±	—	—(+)	—	—	—	—	—	(+)			
30	+	—	(+)—	—								
40	+	—	+—									
50	—	—	—									
60		—	—									
70		—										
Animals died during experiment	1	0	1	4	0	0	0	0	0	0	2	1 (included)

* BP — benzo[a]pyrene

§ No signs of the injected oil

TABLE 3 b

Experiments with dibenz[a h]anthracene in various solvents

Experiment No	IV		V		VI		XV		XVI	
Substance	DBA* in pea nut oil	Pea nut oil	DBA in pea nut oil	Pea nut oil	DBA in pea nut oil	Pea nut oil	DBA in olive oil	Olive oil	DBA in peanut oil	Peanut oil
Number of animals (Triton cristatus)	3	9	9	9	9	9	7	14	7	7
Epidermal reaction on										
3 day							—§	see under MCA		see under MCA
6 7	+		+	-	—	—	±		(+)	
9 10	+	—		—	+	—	+		±	
12							+		+	
15	+		+	-	+	—	+		+	
18							+		(+)	
20 21	+		+	—	+	—	(+)			
30	+		(+)		±	—				
40		—	—	—	+	—				
50			-		—	—				
60		—			—					
70			—	—	+	—				
Animals died during experiment	0	0	1	1	0	1	0	0	3 (1 included)	1 (included)

* DBA = dibenz[a h]anthracene
§ No signs of the injected oil

TABLE 3 c

Experiments with 3 methylcholanthrene in various solvents

Experiment No	I		II		III		XI		XII		XV		XVI	
Substance	MCA* in peanut oil	Peanut oil	MCA in peanut oil	Peanut oil	MCA in peanut oil	Peanut oil	MCA in peanut oil	Peanut oil	MCA in soya bean oil	Soya bean oil	MCA in olive oil	Olive oil	MCA in peanut oil	Peanut oil
Number of animals (Triton cristatus)	9	9	9	9	9	9	7	7	7	7	7	14	7	7
Epidermal reaction on														
3 days							—	—	—	+	—	— —	+	—
6–7 –	—	—	+	—	—	—	(+)	—	+	—	—	± —		—
9–10 –	—	-	-	—	+	—	+	—	+	—	+	— —	—	—
12							—	—	(+)	—	(+)	— —	—	—
15	+	—	+	—	—	—	+	—	+	—	+	— —	+	—
18							+	—	+	—	+	— —	—	—
20–21	—	—	—	-	—	—	—	—	—	—	+	— —	—	—
30 –	+	—	+	—	+	—								
40 –	—	—	+	—	—	—								
50 –	—	—	—	—	—	—								
60	—	—	—	—	—	—								
70	—	—	—	—	—	—								
Animals died during experiment	0	0	0	0	0	0	0	0	0	0	0	0	1	1 (included)

* MCA = 3 methylcholanthrene

REFERENCES

Arffmann F Heated Fats and Allied Compounds as Carcinogens A Critical Review of Experimental Results J Nat Cancer Inst *25* 893 926 1960

Breedis C Transplantable Sarcoma of the Salamander Induced by Methylcholanthrene Cancer Res *11* 239 1951

Breedis C Induction of Accessory Limbs and of Sarcoma in the Newt (Triturus viridescens) with Carcinogenic Substances Cancer Res *12* 861 866 1952

Champy C H & Mlle Champy Epithelioma transmissible du triton Bull Ass c franc p l etude du cancer *24* 206 220 1935

Fedatov D M Compt rend Acad d sc de l USSR *31* 87 90 1941 (Cit from Hartwell J L Survey of Compounds which have been Tested for Carcinogenic Activity 2nd ed Public Health Service Publication No 149 Bethesda 1951)

Finkelstein E A Uspekhi Sovremennoi Biol *17* 320 1944 (Cit from Lucké B & Schlumberger, H G Physiol Rev *29* 91–126 1949)

Hellmich W Experimentelle Hervorrufung atypischer Epithelwucherungen bei Amphibien Ztschr f Krebsforsch *28* 44 46 1929

Koch C Schreiber B & Schreiber, G Tumeurs produites par le goudron et le benzopyrene chez les amphibiens urodeles Bull Assoc franç p l etude du cancer *28* 852 859 1939

Leoni V Tumori da metilcolantrene in tritoni Ist di Zool e Anat Comp Univ di Milano R C Ist Lombardo Sci Lett (Sci Biol Med) *92*(B) 220 240 1958 (Abstract from Excerpta Medica Cancer 7 818 1959)

Lucké B & Schlumberger H G Neoplasia in Cold Blooded Vertebrates Physiol Rev *29* 91–126 1949

Neukomm S Le problème de la cancérisation par le goudron et les substances cancérigenes chez les Tritons Mémoires de la Société Vaudoise des Sciences naturelles Lausanne *8 137 215 1944*

Neukomm S Un test sensible et ultra rapide du pouvoir cancérigene de certaines substances chimiques Oncologia *10* 107 119 1957a

Neukomm S Recherches expérimentales sur le pouvoir cancerigène de la fumée du tabac et d autres polluants de l atmosphere Oncologia *10* 137 156 1957b

Neukomm S The Newt Test for Rapid Biological Assay of Certain Carcinogenic Substances Acta Unio internat contra cancrum *15* 654 658 1959

Neukomm S & Luder Huguenin M Investigations on the Neoplastic Power of Chemical Compounds in Newt Oncologia *13 294 303 1960*

Pflugfelder O Experimentelle Erzeugung von Epidermiswucherungen bei Amphibien Biol Zentralblatt *68* 96 106 1949

Schlumberger H G & Lucké B Tumors of Fishes Amphibians and Reptiles Cancer Res *8* 657 753 1948

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PRIMARY CARCINOMA ARISING IN CONGENITAL LIVER IN CONJUNCTION WITH MILIARY CHOLANGIOMATOSIS

Report of a Case

By

AKE G H LINDGREN, GORAN HANSSON and LARS-AKE NILSSON

Received 4 1 61

Relatively often the liver is the site of malformation One type of abnormality is the cystic liver which in some instances is associated with cysts in other organs, particularly in the kidneys and pancreas Usually a cystic liver has no clinical significance

In a recently observed case of primary carcinoma in a cystic liver in conjunction with miliary cholangiomatosis the carcinoma had evidently arisen from the malformations Since the cystic liver in this instance had a clinical significance the case seemed to warrant description

CASE REPORT

History

In 1948 a 74 year old man was admitted to hospital complaining of difficulty with urination which on examination was diagnosed as hypertrophy of the prostate gland At that time bilateral vasectomy and transvesical [illegible]

[illegible] the contrast agent urographic investigation could not be performed and the origin of the hematuria could not be clearly determined Three to four years later the hematuria returned but he did not seek medical advice

In January 1960 he complained of fatigue shortness of breath and anorexia Thereafter he lost about [illegible] kg in weight [illegible]

[illegible] mg [illegible] prothrombin level 89% [illegible] and serum protein 6.7 g% Electrophoretic analysis showed a reduced albumin fraction and increased levels of the alfa and gamma globulin fractions The urine sediment showed an abundance of white blood cells and 1 2 red cells per counting area as well as a large number of bacteria Roentgenograms of the colon

the stomach and the lungs showed no significant changes Abdominal paracentesis was performed and 2800 ml of clear, yellowish fluid was withdrawn Microscopic examination showed a large amount of sediment which was composed of almost equal parts of red blood cells and large mononuclear cells similar to the type which cover the peritoneal cavity No malignant-suspect cells were encountered Histological examination of biopsy material obtained by liver puncture showed a picture of a low differentiated scirrhous adenocarcinoma, which was supposed to be metastatic After laparocentesis and treatment with diuretics there was general improvement The patient was discharged but continued to receive out-patient treatment with increasing ascites and oedema of the legs Abdominal paracentesis was performed and 5400 ml of clear straw-colored ascitic fluid were removed Hardly three weeks later he vomited a large quantity of blood and expired a few days later with symptome of shock due to blood loss

Autopsy

The abdominal cavity contained from 2–3 litres of clear yellow fluid The entire peritoneum was smooth and glistening, and no tumor indications were apparent The stomach was filled with blood but the mucous membrane showed only post-mortem changes Collapsed varicose veins were observed in the lower third of the oesophagus A considerable quantity of blood was also found in the intestines The liver was *enlarged and weighed about 2000 g On the surface of the liver and* diffusely spread throughout the parenchyma a large number of well-defined, occasionally confluent, white, solid tumours were found Some of these were as large as a plum, and contained no hemorrhagic or necrotic tissue Centrally located within some of the tumours and often juxtaposed to them were cysts which varied in diameter from 5 to 10 mm The contents of the cysts were liquid and clear Throughout the remaining portions of the liver there were numerous retracted solid grey tumours of rice-grain size and in the parenchyma as well as in proximity to these tumours some peppercorn sized cysts were observed The spleen was enlarged (500 g) and the surface of the cut section was dark *red The intestines and pancreas* were without pathological changes The kidneys showed signs of pyelonephritic shrinkage The bladder had a trabeculated wall but the mucous membrane was normal The prostate gland was somewhat enlarged by relapse of adenomatous hyperplasia No cysts or *pathological changes were encountered in the other organs*

Histological Examination

The larger tumours presented a picture of adenocarcinoma which had destructively infiltrated the liver parenchyma In the periphery of the cancer tumours the stroma was as a rule sparse while in many instances in the more central portions an abundant and in some areas *hyalinized* stroma was found (Probably the liver biopsy material originated from such a relatively scirrhous portion) Within the malignant tissue numerous cysts were found which were of varying size and had a low cubical epithelium In the non-malignant portions of the liver, cysts were also *found but not in such great numbers* as within the cancer tumours Usually these cysts lay in conjunction with adeno-



Fig 1
Adenomatous bile duct proliferations with cubical epithelium and embedded in connective tissue (Miliary cholangioma)

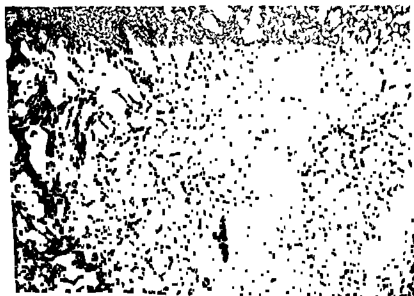


Fig 2
Transformation from adenomatous bile duct proliferations to scirrhous cancer simplex



Fig 3.



Fig 4

Figs 3 and 4 Liver cysts with surrounding adenomatous bile duct proliferations with transformation to adenocarcinoma and cancer simplex

matous bile duct proliferations which had a low cubical epithelium (Fig 1) The lumina of the ducts were cell free some of them showed a low protein content and some ducts contained more or less strongly colored bile Within the smaller tumours a normal bile duct was found which contained regular somewhat high columnar epithelium and which passed near branches of the hepatic artery and portal vein In several places transitional forms between the adenomatous bile ducts and carcinoma were observed (Figs 2–4) The walls of many of the bile ducts which passed through the rounded tumours had a regular epithelium while adjacent ducts contained proliferating atypical epithelium of the type associated with carcinoma The liver parenchyma was otherwise of normal structure and no indications of blood stasis were evident In the peripheral portions of the liver lobuli moderate fatty degeneration of the cells was observed There was no increase of the connective tissue with the exception of those areas where the cysts and the adenomatous bile duct proliferations were situated

DISCUSSION

In cases of cystic liver the entire parenchyma is irregularly permeated by cysts af varying size which are readily observed macroscopically (*Herxheimer* 1930) The etiology and hereditary factors associated with cystic liver have not been conclusively clarified According to certain authors the cystic dilatation may arise from discontinuities of the embryonic bile ducts (among others *v Meyenburg* 1917) In the present case the cysts were only sparsely distributed in the unchanged portions of the liver while the carcinomatous tissue was replete with cysts some in the vicinity of the malignant tumours and others within them *Lindgren & Ranström* (1953) reported a change in the liver which was closely related to cystic liver and which they referred to as miliary cholangiomatosis In this case miliary nodules formed of adenomatous bile duct proliferations embedded in connective tissue were observed Occasionally these miliary nodules are found in the conjunction with cysts in cases of cystic liver The authors pointed out that serial sections of the liver demonstrated that the adenomatous formations communicated with the surrounding liver parenchyma but not with the normal bile duct passing through the tumour In the present case there are also many indications of miliary cholangiomatosis however the concomitant cysts require the diagnosis of cystic liver

In the literature we have only found one case resembling the one described here (*Willis* 1943) This was a 27 years old woman with a multicentric bile duct carcinoma arising from the epithelium of cysts in a congenital cystic liver At autopsy metastases were demonstrated in the regional lymph glands and the lungs The left lobe of the liver was almost completely replaced by cysts and tumor tissue The remainder of the liver parenchyma contained numerous globular meta

stases with a maximum diameter of 5 cm The histological findings indicated that the tumor was a well differentiated slightly papillary adenocarcinoma No cysts were found in the other organs

In the present case it is possible to show transformation from benignant cholangiomatous nodules in the cystic liver to adenocarcinoma *Evidently, the cancer was primarily multicentric in origin similar to the* hepato-cellular cancer in Laennecs cirrhosis and also similar to some cases of bile duct cancer No distant metastases were found in this case

While alive, the man showed signs of splanchnic hypertension with ascites, varicose veins in the oesophagus and splenomegaly His death was due to hemorrhage from these varicose veins The absence of cirrhotic changes in the parenchyma of the liver indicated that the splanchnic hypertension had been caused by pressure of the tumour tissue on the branches of the portal vein The absence of blood stasis within the liver indicates that there had been no obstruction of the hepatic veins

SUMMARY

A case of a 74 year old man with multicentric liver carcinoma in conjunction with cystic liver is described Histological examination showed transformation from benignant adenomatous bile duct proliferations of the cystic liver (miliary cholangiomatosis) to adenocarcinoma No extra-hepatic metastases were observed The clinical picture was one of splanchnic hypertension with ascites and lethal bleeding from ruptured varicose veins of the oesophagus

REFERENCES

1 *Herxheimer G* Henke Lubarsch s Handb d spez Path Anat 5 *I* 797 1930
2 *Lindgren Alf G H & Ranstrom S* Miliary cholangiomatosis of the liver Acta path et microbiol scandinav 33 159 1953
3 *v Meyenburg H* Über die Cystenleber Beitr path Anat *64* 477 1917 18
4 *Willis R A* Carcinoma arising in congenital cysts of the liver J Path Bact 55 492 1943

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ANTIBODY RESPONSE TO INFLUENZA A_2 AND A_1-STRAINS EXAMINED WITH VIRUSES OF DIFFERENT ANTIBODY SENSITIVITY

By

ARILD HARBOE

Received 23.xii.60

In a previous investigation (7) the authors examined the dynamics of the development of haemagglutination inhibiting (HI) antibodies in ferrets that had been infected with influenza virus of the A_2-type. It was found that when the HI test was performed with virus of a low antibody sensitivity (a.s.) the peak titre as a rule was recorded several days later than when virus of a high a.s. was used, irrespectively of the a.s. of the infecting virus. This phenomenon was observed only in animals that had not been exposed to A_2-virus before.

In the previous investigation (7) the animals had been infected by intranasal instillation of infective egg allantoic fluid. Among the problems dealt with in the present publication are the HI-antibody development in ferrets and humans infected spontaneously or vaccinated with A_2, and the development of infectivity neutralizing antibodies in A_2-infected ferrets.

The HI antibody development in infected ferrets was also studied in the light of the observation by *Styk & al.* (18, 19) of thermolabile A_2-antibodies. In other experiments the development rates of A_1-antibodies were tested with P and Q lines of these strains in order to see if conditions were analogous to those found within the A_2-type. The paper also describes attempts at electrophoretic separation of antibodies with preference for viruses of different a.s., and attempts at increasing the a.s. by means of trypsin treatment.

MATERIALS AND METHODS

Virus strains. Details about the A_2 strains [illegible]

[illegible] and subsequently had acquired a [illegible] strains had been received from Dr. Isaacs and Dr. Stedmyr

Antibody tests The HI test was performed the same way as previously (7) As a routine the sera were pretreated with cholera filtrate (20) The tests were put up in Perspex plates and the patterns observed (12) The HI titre was given as the reciprocal of the primary serum dilution (before addition of other reagents) which partly inhibited 4 haemagglutinating doses of the antigen

The infectivity neutralization test was performed in eggs 10 to 11 days old The sera were inactivated for ½ hr at 56° C and serial twofold dilutions incubated for one hour in ice water with a constant amount of virus The eggs were inoculated intra allantoically with 0 1 ml of the serum virus mixtures and two days afterwards tested on the presence of haemagglutinins

Electrophoretic fractionation of sera was performed on starch grain according to the method of *Kunkel & Slater* (1?) with minor modifications (8)

RESULTS

1 *Ferret Sera*

Development and resistance of the HI antibodies after A_2 infection Previously (7) the ferrets had been infected under ether anaesthesia by intranasal instillation of one ml of undiluted virus infected egg allantoic fluid In the following experiment it was examined if the difference between the antibody development rates was demonstrable also in ferrets that had been infected through natural transmission This type of infection took place when two uninfected animals *I* and *J* were transferred to a cage which housed two animals *G* and *H* that the day before had been inoculated intranasally with allantoic fluid from eggs infected with the egg ferret mouse egg line (EFME) of A Japan/305/57 The transmission of the infection to ferrets *I* and *J* was assumed to take place about three days after their cagemates had been inoculated

The influence of the pretreatment of the sera was examined in the same experiment The sera were tested a) in the active state b) after heating for ½ hr at 56° C c) after treatment with *cholera filtrate over night* at 37° C followed by heating for 1 hr at 56° C The active sera were tested at 4° C in order to avoid haemolysis

All sera were tested against two viruses of low ? s (A₂/Japan/305/57 egg line (E) and A_2/Norway/2 57) and against two viruses of high ? s (A_2/Japan 305/57 EFME and A_2/Rockefeller Institute/5 57) Typical results are presented in Table 1 The other two ferrets *H* and *J* gave results essentially identical to ferrets *G* and *I* neither was there any distinct difference between the results obtained with the two strains of the same ? s

It was observed that when viruses of a high ? s were used as antigens in the test regardless of the way the sera had been pretreated *the titre peak was recorded in the first of the convalescent samples while in the subsequent samples a gradual decline was observed* When viruses of a low ? s were used as antigens the titre of the first convalescent sample when tested in the active state was of the same magnitude as the titre of the subsequent sample Treatment with cholera filtrate caused a heavy reduction of the titre of the first sample but only a minor reduction of the subsequent Experiments performed in order to examine the effect of simple heating gave less distinct results

TABLE 1

	Number of days after infection	Active sera		Sera heated for 1/2 hour at 56° C		Sera treated with cholera filtrate and heated for 1 hour at 56° C	
Ferret G (inoculated intranasally)	0	< 6	24	< 12	24	< 6	< 6
	10	48	1600	36	2400	< 6	1600
	20	48	800	–		24	1200
	30	36	400	36	600	36	800
	227	24	150	18	150	24	200
Ferret I (infected naturally)	12	48	1200	12	1600	< 6	1600
	22	48	600	24	800	24	800
	32	24	200	24	200	24	600
	225	12	100	12	100	12	150
	Test virus	N/2	RI/5	N/2	RI/5	N/2	RI/5

These findings suggest, that the antibody reacting with virus of low a s was less resistant to the treatments in the early sample than in the later samples, and less than the antibody reacting with virus of high a s in any of the samples On the other hand it should be noted that in

so this animal obviously had developed the more permanent type of antibodies already after 10 days

Table 1 also demonstrates that between the two modes of infection there was no difference as to the dynamics of antibody development This was confirmed with inactivated and with cholera treated sera from another two ferrets, one of them dripped intranasally and the other infected naturally by the former, bled 10 20, 30 40 and 72 days after the inoculation and tested simultaneously with eleven viruses of high and nine of low a s

The development of infectivity neutralizing antibodies after A_2-infection Two groups of experiments were performed 1) A_2 Japan/305 57 F and I I MI (low and high a s) were tested against pools of equal amounts of sera obtained from three ferrets 14 and 28 days after intra nasal instillation of A_2 Singapore 1/57 2) A_2 Norway/2 57 (low a s) and A Norway 9 57 (high a s) were tested against pools of equal amounts of sera obtained from two ferrets 13 and 23 days after natural exposure to A_2 Japan 305 57 FFMI

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with le [illegible] presented in Table 2 It is seen that the

28 days pool is neutralizing the line of low a.s. more than the 14 days pool does, while there is practically no difference between 14 and 28 days as regards the neutralizing activity against the line of high a.s. An HI-test with the two anti Singapore serum pools gave the following values: Against Japan LFME after 14 days 1200, after 28 days 600. Against Japan E after 14 days 12, after 28 days 36.

TABLE 2

Egg Infectivity Neutralization Test on Ferret Sera drawn after Infection with A_2/Singapore/1/57 and Examined with Virus Lines of different Antibody Sensitivity. 1/5 Means that one out of five Eggs Developed Haemagglutinins

Tested with	Serum dilutions	Samples drawn after		
		0 days	14 days	28 days
	10	4/4	1/5	0/4
200 ID_{50} of	20		6/7	1/7
A_2/Japan/305/57	40		7/7	3/7
egg line	80		5/5	[illegible]/7
(low antibody sensitivity)	160		4/5	5/5
	320		4/4	4/4
300 ID_{50} of	50	5/5	0/5	0/5
A_2/Japan/305/57	100		1/5	1/5
egg ferret mouse egg line	200		2/5	4/5
(high antibody sensitivity)	400		5/5	5/5

TABLE 3

Haemagglutination Inhibition Titres of Sera from Ferrets Vaccinated with Different A_2 strains. Sera Tested with Viruses of Different Antibody Sensitivity

Ferret given a subcutaneous injection of one ml of	Number of days after the injection	Native sera	Sera treated with cholera filtrate	
	0	< 9	< 9	< 6
A_2/Singapore/1/57	8	9	9	72
(titre 30 000)	15	27	18	96
	22	27	18	36
	0	< 9	< 9	< 6
A_2/Norway/2/57	7	< 9	9	48
(titre 6 000)	14	18	36	96
	24	27	36	48
	62	18	18	18
	0	< 9	< 9	< 6
A_2/Norway/9/57	7	< 9	< 9	24
(titre 8 000)	14	36	36	192
	21	27	36	72
	28	18	36	48
Tested with A_2/Japan/305/57		egg line		egg ferret mouse egg line

The development of HI antibodies after A_2 vaccination. In this experiment the HI antibody development was studied in ferrets that had received a subcutaneous injection of one ml of a concentrated influenza

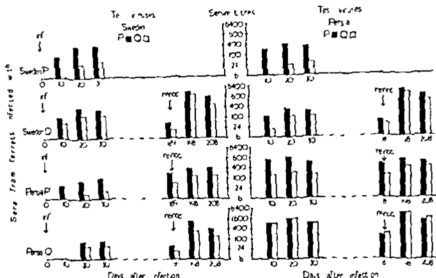


Diagram 1

Cross haemagglutination inhibition test on sera from ferrets infected with various A_1 viruses

A_2-virus preparation When administered by this route an infection is not likely to develop (4) Some results obtained with active and with cholera treated sera from three of the animals are presented in Table 3 They had been given rather heavy amounts of virus in order to produce an antibody response measurable also with virus of low a s For comparison it is noted that these animals received between 2 and 10 times the amount of virus haemagglutinin required in one ml of an American or British influenza vaccine preparation at present

The following conclusions were based on tests with cholera treated sera from seven, subcutaneously injected ferrets, using at least two test strains of high and two of low a s It was found that the maximum titres were reached practically at the same time for the different test strains

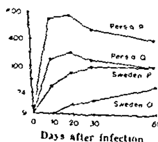


Diagram 2

Haemagglutination inhibition test on sera from the ferret (Diagram 1) infected with A_1 Persian Gulf 2 52, P line

28 days pool is neutralizing the line of low a.s. more than the 14 days pool does, while there is practically no difference between 14 and 28 days as regards the neutralizing activity against the line of high a.s. An HI-test with the two anti-Singapore serum pools gave the following values. Against Japan EFME after 14 days 1200, after 28 days 600. Against Japan E after 14 days 12, after 28 days 36.

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Egg Infectivity Neutralization Test on Ferret Sera drawn after Infection with A_2/Singapore/1/57 and Examined with Virus Lines of different Antibody Sensitivity. 1/5 Means that one out of five Eggs Developed Haemagglutinins

Tested with	Serum dilutions	Samples drawn after		
		0 days	14 days	28 days
	10	4/4	1/5	0/4
200 ID_{50} of	20		6/7	1/7
A_2/Japan/305/57	40		7/7	3/7
egg line	80		5/5	6/7
(low antibody sensitivity)	160		4/5	5/5
	320		4/4	4/4
300 ID_{50} of	50	5/5	0/5	0/5
A_2/Japan/305/57	100		1/5	1/5
egg ferret mouse egg line	200		2/5	4/5
(high antibody sensitivity)	400		5/5	5/5

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Haemagglutination Inhibition Titres of Sera from Ferrets Vaccinated with Different A_2-strains. Sera Tested with Viruses of Different Antibody Sensitivity

Ferret given a subcutaneous injection of one ml of	Number of days after the injection	Active sera	Sera treated with cholera filtrate	
	0	< 9	< 9	< 6
A_2/Singapore/1/57	8	9	9	72
(titre 30 000)	15	27	18	96
	22	27	18	36
	0	< 9	< 9	< 6
A_2/Norway/2/57	7	< 9	9	48
(titre 6 000)	14	18	36	96
	24	27	36	48
	62	18	18	18
	0	< 9	< 9	< 6
A_2/Norway/3/57	7	< 9	< 9	24
(titre 8 000)	14	36	36	192
	21	27	36	72
	28	18	36	48
Tested with A_2/Japan/305/57			egg line	egg ferret mouse egg line

The development of HI antibodies after A_2 vaccination. In this experiment the HI-antibody development was studied in ferrets that had received a subcutaneous injection of one ml of a concentrated influenza

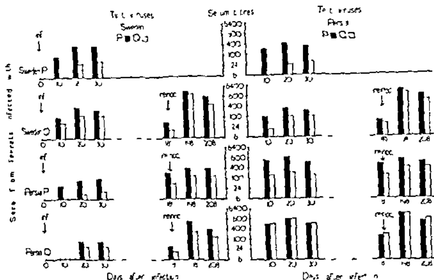


Diagram 1
Cross haemagglutination inhibition test on sera from ferrets infected with various A_1 viruses

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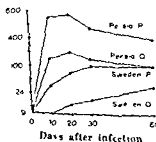


Diagram 2
Haemagglutination inhibition test on sera from the ferret (Diagram 1) infected with A_1 Persian Gulf/2 52 P line

TABL

Haemagglutination Inhibition Test on A2 Convalescent Fer... and Examined with Viruses

Serum fraction no	Unfractionated serum	Haemagglutinat...				
			Gamma globulin			
		1	2	3	4	5
Active	36	< 1	< 1	2	6	1
Active	960	< 1	2	16	32	8
Treated with cholera filtrate	240	< 2	4	12	32	6

Some of the animals revealed a tendency towards a more rapid fall in titres against the test strains of high a s , a phenomenon which regularly was observed in infected animals

The development of HI-antibodies after A_1-infection The ferrets were infected by intranasal instillation as above P and Q-viruses were used for infection as well as for testing The sera were pretreated with cholera filtrate The results are shown in Diagram 1 No distinct difference is observed between P and Q variants of the same strain when used as test antigens to record the time of the highest antibody titre This finding differs from the experience made with the A_2-infected ferrets Another observation of interest is the distinct loss of homologous strain specificity of the Persia P and Q sera which took place as time passed after the infection In order to visualize this particular point Diagram 2 was prepared It is also noted that hyperimmunization did not result in any further broadening of the specificity These observations were confirmed with sera from another two ferrets immunized with Persia P and Q

Electrophoretic serum fractionation This experiment was an attempt to separate antibodies with different serologic features It was examined in HI-tests whether the fractions obtained differed from each other as to the ratio between their antibody titres obtained with test viruses of high a s and of low

The result of testing serum fractions from a ferret bled 12 days after intranasal inoculation with A_2/Singapore/1/57, is shown in Table 4 It is seen from this table that the fractions obtained did not differ as to the ratio mentioned above, in the following to be referred to as the high low a s ratio, to save space The experiment with this serum was repeated, neither this time a separation was achieved

In another experiment cholera treated fractions of serum obtained from a ferret 21 days after intranasal inoculation with Persia P were tested with Persia P and Q, and with Sweden P and Q The latter virus did not record inhibition in any of the fractions (unfractionated serum titre being only 6) The other three test viruses, however, recorded a practically complete recovery of antibody activity Neither with this

...erum Fractions Obtained by Electrophoresis on Starch Grain, ... Different Antibody Sensitivity

...nhibition titres of								
Beta globulin			Alpha globulin				Albumin	Tested with A_2/Japan 305/57
6	7	8	9	10	11	12	13	
< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	egg line
4	3	12	48	32	48	16	8	egg ferret mouse egg line
< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	egg ferret mouse egg line

serum a separation of antibodies with different serologic qualities was observed.

2. Human Sera

The development of the HI-antibodies after exposure to A_2-virus. Sera were collected in February–May 1960 at suitable intervals after infection and made available to the author through the courtesy of *Dr. Ulstrup*, Ullevål Sykehus. The sera were treated with cholera filtrate and tested against two viruses of low and two of high a.s. The results are shown in Table 5. It is seen that antibodies measured with viruses of different a.s. develop at the same rate.

An attempt to study the high/low a.s. ratio in 80 military recruits vaccinated with A_2-virus at the end of February 1960 (6) failed; of the 14 recruits who probably had not been exposed to this virus before, none developed antibody detectable with the test virus of low a.s.

3. Trypsin Treated Virus

The hypothesis has been put forward (11) that a virus of low a.s. has got its dominant antigen situated more deeply in the virus particle than a virus of high a.s. The following experiments was an attempt to uncover the antigens in a virus of low a.s. by treatment with trypsin. One volume of 2 per cent crystalline trypsin (Trypure "NOVO") in M/15 phosphate buffer pH 7.5 was added to one volume of a red cell eluate of A_2/Japan/305/57 L in normal saline and the mixture kept for about 20 hours at 37° C, subsequently the trypsin was destroyed by heating for one hour at 56° C. Before trypsin treatment the haemagglutinating titre was 1400, afterwards 1000, an unsignificant reduction. An HI test was performed with ferret sera drawn 0, 10, 20 and 30 days after A_2 infection and treated with cholera filtrate. The following titres were recorded: Japan L, untreated virus <6, <6, 24, 36; trypsinized <6, 24, 144, 144; heated with the buffer only <6, 6, 24, 24. Untreated Japan H MIE <6, 1536, 768, 576. When the experiment was repeated, the same, modest titre increases were observed.

TABLE 5

Haemagglutination Inhibition Test on A_2-Convalescent Patients' Sera Examined with Viruses of Different Antibody Sensitivity

Sera from patient	Number of days since fever began	Tested with			
		A_2 Norway 2/57	A Japan 305/57		A_2 Rockefeller Institute 5/57
			Egg line	Egg ferret mouse egg line	
C G born 1891	14	< 9	< 6	6	< 6
	24	9	12	96	192
	42	9	12	96	192
	52	9	12	96	144
S H born 1895	7	< 9	6	12	9
	53	< 9	12	48	72
	74	< 9	18	48	72
B I born 1884	5	< 9	< 6	< 6	< 6
	14	9	24	72	72
	33	36	48	192	288
	55	18	36	96	192
I L born 1891	4	< 9	< 6	< 6	< 6
	15	< 9	< 6	6	< 6
	25	< 9	6	48	72
	53	< 9	6	48	96
H S born 1878	5	< 9	< 6	12	12
	13	144	144	384	768
	23	288	144	576	768
	32	288	144	576	768
H T born 1876	1	< 9	< 6	< 6	< 6
	8	< 9	6	48	48
	18	72	72	384	576
	27	144	144	768	1152
A O born 1923	2	< 9	< 6	< 6	< 6
	8	18	72	96	192
	21	18	48	96	192
	36	18	48	96	192

DISCUSSION

A change in the combining capacity of the antibodies formed during the course of an immunizing process has been observed with various antigens. Thus *Jerne* found (13) that diphtheria antitoxin of low avidity (*i.e.* of a low association constant) was formed in the early stages of immunization; with prolonged immunization the avidity of the antitoxin increased. Analogous conditions were found by *Jerne & Avegno* (14) when they studied phage antibodies, and they raised the question whether the quality of the antibody molecules produced at different stages of immunization improved gradually in such a way that those produced later were capable of attaching more firmly to the antigen, or whether only 2 types of antibody molecules of different combining

powers existed, the later serum samples possessing a higher fraction of the more firmly combining type. A definite answer was not given, but the latter view gave a plausible explanation to their observations. *Brunner & Ward* (1) found that convalescent phase poliomyelitis antibody formed a complex with poliovirus which dissociated at a slower rate than the acute phase antibody-virus complex upon dilution of the antibody.

In the present investigation no attempts were made to obtain absolute or relative measures for the strengths of binding between virus and antibody, neither was there any reference to such work in the available literature on influenza A_2-virus of different antibody sensitivity, though related parameters probably can be calculated (3). However, the results referred to above (13, 14, 1) make it natural to expect an increase of the average association constant between the immunizing antigen and the antibodies formed during the course of an influenza infection or during vaccination with repeated injections.

The following interpretation of the HI-titre values observed in ferrets during the course of an A_2-infection is based on the tentative hypothesis that two different types of antibodies then are developed (perhaps produced by two different cell types). It is further assumed that all or at least the great majority (2, 5) of the haemagglutinating particles of an influenza A_2-virus classified in these studies as one of high a.s. (in literature often designated as "avid" or with the letters P or R) has got a high ratio between its affinity to antibody and its agglutinating capacity (depending on its affinity to the virus receptors of the cell type concerned), while correspondingly a virus of low a.s. ("non-avid", Q) has got a low ratio.

The hypothesis is then put forward that the first antibodies to appear have a rather low affinity to viral haemagglutinin and therefore fail to inhibit the haemagglutination caused by virus of a low a.s. However, the affinity is sufficient to inhibit virus of a high a.s. The production of this type of antibody declines early. In the meantime antibodies of a higher affinity are developed. These antibodies can inhibit virus of low as well as of high a.s., and the production of this type does not decline so early as that of the former. [illegible]
sufficient to compensate fo[illegible]
of the former type and th[illegible]
to drop during the course of the infection. The early antibodies of high affinity can be destroyed by heating at 56° C, and in particular by treatment with cholera filtrate followed by heating. Later in the course of the infection these antibodies become resistant. The antibodies of low affinity are resistant through all stages of the infection. These should be the reasons why with the pretreated sera the decrease with time in the high/low a.s. ratio was greater than with active sera.

It is implicit in the considerations above that the antibodies of both types are liberated into the blood stream as soon as they are produced,

and that they do not differ from each other as to the rate of their destruction or removal from the blood stream.

With A vaccinated ferrets the decrease in the high/low a.s. ratio, if any decrease at all, was less distinct than in infected animals and the antibodies recorded with virus of low a.s. seemed to resist the treatment with cholera filtrate at any stage of immunization. May be that the decrease in the ratio met with in infected ferrets reflects a change of the immunizing agent during the course of the infection and that this change does not, or only to a minor extent, take place under conditions when the virus does not multiply, as when injected subcutaneously.

Unlike sera from A infected ferrets, human convalescent sera treated with cholera filtrate did not show any decrease in the high/low a.s. ratio in the successive samples. Only sera from adult persons were available in all seven patients and except for one they were all more than 65 years old and thus in all probability had experienced a substantial number of influenza infections previously which might have conditioned their antibody response to the present one. An analogous examination of human infants' sera would certainly have been of interest, in particular because there seems to exist species differences as regards the A antibodies. *Styk & al.* (18, 19) demonstrated that in A infected mice during the whole course of the infection titres recorded with virus of low a.s. were markedly reduced when the sera were heated while only the titres recorded with virus of high a.s. were stable under the same conditions. Further, a decrease in the high/low a.s. ratio during the course of the infection did not take place in mice, to judge from the titre values presented. In all these respects vaccinated mice presented the same picture as the infected.

The serum electrophoresis was performed because of reports in literature on different electrophoretic behaviour of the antibodies formed with increasing degree of immunization (for references see (16, 17)). In the latter paper the appearance of two anti-sheep red cell peaks were demonstrated by starch grain electrophoresis in a late immune serum but only one in an early. In the present study a single peak was found and this was situated in the same fractions regardless of the a.s. of the test virus. This result merely means that if more than one type of III antibody is produced, they were indistinguishable by the fractionation method employed here.

In the A_1-antibody development no decrease in the ratio between titres measured with P and Q lines of the respective strains was observed during the course of the infection. The Q lines of the A_1-strains however were not completely analogous to the A viruses of low a.s. because the former gave rather high titres with homologous serum

SUMMARY

Ferrets infected with influenza A_2-virus by natural transmission from cagemates inoculated by intranasal instillation of infective egg allantoic fluid showed the same pattern of the haemagglutination inhibiting antibody response as their inoculated cagemates, provided none of them had been exposed to this virus before As previously reported, characteristic for this response is the decreasing ratio between the titres recorded with virus of high and with virus of low antibody sensitivity (a s) when successive serum samples are compared The possibility was discussed that two types of post-infectious A_2-antibodies might be formed one of low affinity and characteristic of the early phase, the other of high and characteristic of the late An attempt to separate these hypothetical antibody types by electrophoresis and thus prove their existence failed

The decrease in the ratio was less apparent in A_2-vaccinated ferrets In A_2-convalescent, human adults it was completely absent, suitable sera from children were not at hand The decrease was not seen in A_1-infected ferrets, here a reduction of homologous strain specificity was observed with one of the two strains examined

It was found that heating at 56° C, and in particular treatment with cholera filtrate followed by heating, might reduce antibody titres recorded with virus of low a s in ferret sera obtained early after A_2-infection, but not in late samples in none of the samples antibody titres recorded with virus of high a s were reduced With sera from A_2 vaccinated ferrets no reduction was seen

A decrease in the ratio between titres recorded with virus of high and low a s was also met with in infectivity neutralization tests on early and late sera from A_2-infected ferrets

Attempts to convert an A_2-virus of low a s into one of high by means of trypsin treatment resulted in a modest increase of the a s

REFERENCES

1 *Brunner K T & Ward R* Differences in stability of antigen antibody complexes formed with poliovirus and acute and convalescent phase human sera J Immunol *83* 405–410 1959

2 *Choppin P W & Tamm I* Two kinds of particles with contrasting properties in influenza A virus strains from the 1957 pandemic Virology *8* 539 542 1959

3 *Drescher J* Adsorptionsphanomene als Mittel zur Differenzierung von Virusarten Zentralbl Bakt *176* 295–301 1959

4 *Francis T Jr* Quantitative relationships between the immunizing dose of epidemic influenza virus and the resultant immunity J Exper Med *69* 283 300 1939

5 *Fridman F A* The heterogeneity of type A2 influenza virus population Acta virol *4* 274 292 1960

6 *Harboe A B thne B & Berg K* to be published

7 Harboe A & Reenaas R The course of antibody development to influenza A_2 strains Examined in the haemagglutination inhibition test with viruses of different sensitivity to homologous antibody Acta path et microbiol scandinav *46* 266 272 1959

8 *Harboe A Reenaas R & Oppedal M* Studies on Francis inhibitor of influenza virus haemagglutination in rabbit serum fractions obtained by electrophoresis on starch grain Acta path et microbiol scandinav 44 92 105 1958
9 *Isaacs A* P Q variation in influenza viruses Lancet 676 678 1953 i
10 *Isaacs A & Andrewes C H* The spread of influenza Evidence from 1950 1951 Brit M J 921 927 1951 ii
11 *Isaacs A Depoux R & Fiset P* The viruses of the 1952 3 influenza epidemic Bull World Health Organ *11* 967 979 1954
12 *Isaacs A Gledhill A W & Andrewes C H* Influenza A viruses Laboratory studies with special reference to European outbreak of 1950 1 Bull World Health Organ *6* 287 315 1952
13 *Jerne N K* A Study of avidity based on rabbit skin responses to diphtheria toxin antitoxin mixtures Acta path et microbiol scandinav *Suppl 87* 1951
14 *Jerne N K & Avegno P* The development of the phage inactivating properties of serum during the course of specific immunization of an animal Reversible and irreversible inactivation J Immunol *76* 200 208 1956
15 *Kunkel H G & Slater R J* Zone electrophoresis in a starch supporting medium Proc Soc Exper Biol & Med *80* 42 44 1952
16 *Laurell A -B* On antibodies separated by paper electrophoresis with special reference to the Wassermann reagins Acta path et microbiol scandinav *Suppl 103* 1955
17 *Stelos P & Taliaferro W H* Separation of antibodies by starch zone electrophoresis Analyt Chem *31* 845 848 1959
18 *Styk B Rathova V & Blaškovič D* Thermolability of specific haemagglutination inhibiting antibodies against the IF influenza virus and their reactivation by the addition of fresh serum Acta virol *2* 179 187 1958
19 *Styk B Kocišková D & Blaškovič D* Dynamics of immunological response to infection with Asian type (A2) influenza virus strains Acta virol *3* *(Suppl)* 97 106 1959
20 *Veen J v d & Mulder J* Studies on the antigenic composition of human influenza virus A strains with the aid of the haemagglutination inhibition technique Onderz Meded Inst Praevent Gen No 6 Leiden 1950

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DISINTEGRATION BY FREEZE PRESSING

2. Effects on Fungi and Phages

By

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Received 6.iii.61

As shown in preceding reports (*Edebo* 1960, 1961) a great variety of cells are disintegrated when, in frozen state, they are forced (2000 atm., −25° C) through the small hole in the X press. The earlier studies were mainly concerned with bacteria. In the present paper it will be demonstrated that other microorganisms tend to be disintegrated similarly.

MATERIAL AND METHODS

[illegible] cial material) was washed [illegible] mately 0.07 M, pH = 7.35) [illegible] = 4.5 × 10⁹ cells/ml). The [illegible] frozen down and stored at −25° C until used.

The total count was determined with the aid of a phase contrast microscope and [illegible]

[illegible] coli B grown in [illegible] repeated differ[illegible] counts were made in the usual way (*Adams* 1959).

The disintegrations of the fungi were performed in the large X press at −25° C and the phages were treated in the small X press at −60° C.

The [illegible] rep[illegible]

RESULTS

There is a clear cut increase in the degree of disintegration of yeast cells as a consequence of repeated pressings. Identical experiments are illustrated in both Table 1 and Fig. 1. The decrease in the viable count

This work was made possible by a grant from the *Wallenberg Foundation*. The technical assistance provided by Miss *Ulla Spet* has been highly appreciated.

[illegible] kindly supplied by Dr. *H. Markkula*.

8 *Harboe A Reenaas R & Oppedal M* Studies on Francis inhibitor of influenza virus haemagglutination in rabbit serum fractions obtained by electrophoresis on starch grain Acta path et microbiol scandinav 44 92 105 1958

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DEPARTMENT OF BACTERIOLOGY, KAROLINSKA INSTITUTET, STOCKHOLM 60, SWEDEN (HEAD PROFESSOR B MALMGREN)

DISINTEGRATION BY FREEZE-PRESSING

2 *Effects on Fungi and Phages*

By

L EDEBO

Received 6 iii 61

As shown in preceding reports (*Edebo* 1960, 1961) a great variety of cells are disintegrated when, in frozen state, they are forced (2000 atm, —25° C) through the small hole in the X-press The earlier studies were mainly concerned with bacteria In the present paper it will be demonstrated that other microorganisms tend to be disintegrated similarly

MATERIAL AND METHODS

Saccharomyces cerevisiae (200 g wet weight commercial material) was washed twice in 800 ml cold citrate phosphate buffer (approximately 0 07 M pH = 7.35) and suspended in citrate phosphate buffer (total count ≈ 4 5 × 10^9 cells/ml) The suspension was distributed in Petri dishes frozen down, and stored at —25° C until used

The total count was determined [illegible]
a Buerker blood cell co[illegible]
after dilution in broth [illegible]

[illegible] Plaque counts were made in the usual way (*Adams* 1959)

The disintegrations of the fungi were performed in the large X-[illegible]

RESULTS

There is a clear-cut increase in the degree of disintegration of yeast cells as a consequence of repeated pressings Identical experiments are illustrated in both Table 1 and Fig 1 The decrease in the viable count

This work was made possible by a grant from the *Wallenberg Foundation* The technical assistance provided by Miss *Ulla Spetz* has been highly appreciated
[1] Kindly supplied by Dr *H Markkula*

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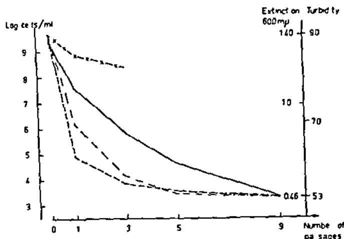


Fig 1
The alteration of viable count (———) total count (+ +) extinction 600 mμ ( ) and turbidity ( ) when yeast cells are pressed repeatedly

is considerable but it is much less marked in the total count Estimation of disintegration by Gram stained smears suggests the process to be even less efficient if well stained cells are considered to correspond to viable cells and cells without damages observable in the phase contrast microscope However some of the well stained yeast cells from repeatedly pressed samples have vague boundaries and destruction of the cell walls can be seen frequently Both the light and electron photo micrographs show that all stages of disintegration of the cells exist from a small limited affection of the cell wall (Fig 2 3 8,9) to a more or less complete disintegration (Fig 6 12) In the phase contrast photo micrographs many subcellular particles are observed in the preparations from material pressed once (Fig 3), but the number sharply decreases in preparations from materials pressed many times (Fig 4 5 6) The particles are strongly refractive and Gram positive but they do not stain with Lugol s iodine

Both the turbidity and the extinction of the suspensions are reduced when the material is pressed repeatedly The reduction obtained in the fourth to the ninth pressings is however not so great as that obtained in the first three pressings The soluble material released chiefly of protoplasmic origin is increased with every pressing as indicated by the step-wise increase in both soluble nitrogen and the ultraviolet absorption of the supernatant samples Again the increase in soluble material in the fourth to the ninth pressings is small compared with that which follows the first three pressings The viscosity of the pressed suspensions increases with every treatment However the change is most pronounced in the first few pressings

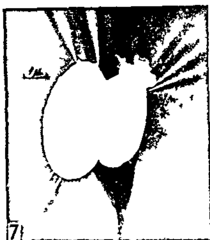
As seen in Fig 13 Aspergillus mycelia are disrupted almost com

TABLE 1

Disintegration of Saccharomyces cerevisiae

The column with mgN/ml usually shows the nitrogen contents of the supernatants but the value in brackets shows the nitrogen content of the whole suspension

Number of passages	Viable count (cells/ml)	Total count (cells/ml)	Gram stained smears	Extinction [illegible] mμ Dil 1:100	Nephelometric value Dil 1:100	Specific viscosity η_{sp} Dil 1:5	mg N/ml (supern.)	Extinction (supern.) Dilution 1:200	
								260 mμ	280 mμ
0	3.0 × 10^9	4.6 × 10^9	–	1.40	91	0.12	0.45 (7.00)	0.057	0.034
1	3.8 × 10	7.8 × 10^8	+++	0.71	70	0.30	4.15	0.550	0.290
3	6.9 × 10^5	2.3 × 10^8	+++	0.54	58	0.34	4.59	0.590	0.320
5	4.3 × 10^4		++++	0.50	54	0.36	4.64	0.660	0.340
9	2.1 × 10^3		++++	0.46	53	0.36	4.68	0.660	0.340











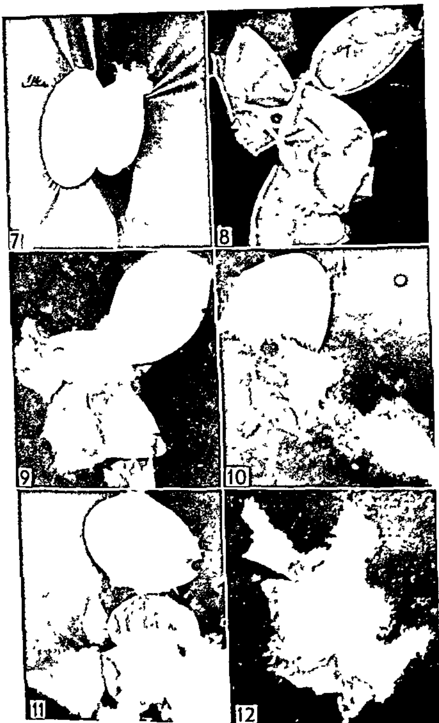


Figs 7-12

Electron photomicrographs of untreated yeast cells (7) and cells pressed once (8) three times (9) five times (10) and nine times (11, 12)



Figs. 2–6
Phase contrast photomicrographs of untreated yeast cells (2) and cells pressed once (3) three times (4) five times (5) and nine times (6)



Figs 7 12

Electron photomicrographs of untreated yeast cells (7) and cells pressed once (8) three times (9), five times (10) ...



Fig 13
Mycelium of Aspergillus oryzae pressed once
(Phase contrast photomicrograph)

Fig 14
Penicillium conidia pressed three times
(Phase contrast photomicrograph)

pletely by a single pressing Upon breaking of the cell walls many particles are released

Conidia from different species of Penicillium have been treated in the X-press About 50 per cent of them are disrupted by one pressing A photograph is chosen of conidia pressed three times (Fig 14) It reveals a higher degree of disintegration, but the cell walls still retain the shape of the whole cell The addition of 0 01 per cent sodium lauryl sulphate, 0 01 per cent cetyltrimetylammonium bromide or 0 01 per cent gelatine, in an attempt to decrease the surface tension and bring water into closer contact with the very hydrophobic conidial surface, does not appreciably affect the disintegration effect

When bacteriophages are pressed, the number of plaques obtained after dilution and plating is reduced (Table 2) Electron microscopical observations on phage material pressed once (Fig 15) show disrupted phage heads, most frequently connected to their tails but also many separate tails and tail fragments These and other observations are similar to those made by *Williams & Fraser* (1956) after freezing and thawing of T2 phages, but the separation of the tail sub-units obtained by freezing and thawing (*Williams & Fraser* 1956) or by oxidizing agents (*Kellenberger & Arber* 1955) is not seen here



Fig 15
Electron photomicrograph of T 2 phages pressed once

TABLE 2
Pressing of T 2 phages
Reduction of the number of plaque yielding phages by different numbers of pressings
The different experimental series were performed on the same phage batch

Treatment	Plaque yielding units ml		
	Series 1	Series 2	Series 3
None	$1\ 4 \times 10^{10}$	$1\ 3 \times 10^{10}$	$1\ 1 \times 10^{10}$
Frozen	$5\ 4 \times 10^{9}$	$4\ 1 \times 10^{9}$	$5\ 8 \times 10^{9}$
1 pressing	$1\ 2 \times 10^{5}$	$8\ 6 \times 10^{6}$	$9\ 4 \times 10^{6}$
3 pressings	$3\ 1 \times 10^{2}$	$2\ 8 \times 10^{2}$	$1\ 2 \times 10^{3}$
5 pressings	< 10	$1\ 3 \times 10^{3}$	$9\ 0 \times 10^{3}$
9 pressings	$1\ 5 \times 10^{3}$	$1\ 5 \times 10^{3}$	~ 2

DISCUSSION

If the disintegration of yeast cells is estimated by viable count, it seems to be much greater than the disintegration estimated by total count and Gram stained smears A likely explanation, which is supported by the morphological data, may be that some cells are damaged without dissolution of the protoplasm Experiments in which the pressed cells were kept in an ice water bath for lengths of time up to 20 hours before plating (either undiluted in the original citrate-phosphate buffer or diluted in broth) did not give large variations in viable count Thus

no repair of slight damages has taken place as occurred with heated bacteria (*Hedén & Wyckoff* 1949). An increase of 20 per cent in viable count was noted in the undiluted material after four hours or more, and at corresponding times, a 10 per cent decrease was noted in the suspensions diluted in broth. The great differences between viable counts, total counts and Gram-stained smears are similar to the results obtained when Streptococcus faecalis were pressed (*Edebo* 1961) where killing effects short of cell wall disruption could not be excluded

The reduction in turbidity and extinction after pressing yeast cells is much less pronounced than that obtained with most bacteria (*Edebo* 1961). This may be due to the great difference in composition and the greater thickness of the yeast cell wall. Yeast cell walls contain, among other things, about 30 per cent glucan which is highly insoluble in water and responsible for the shape of the cell (*Northcote & Horne* 1952). The cell wall thickness is about 0.16 μ, if the cell wall makes up 15 per cent of the cell and the diameter of the cell is estimated at 6 μ The cell wall of yeasts is thus much thicker than that of bacteria (E coli 25 mμ; *Mitchell* 1959), which is also evident from the electron photomicrographs where the yeast cell walls are seen to be very electron-dense (Figs. 8-12).

TABLE 3

Disintegration of Saccharomyces cerevisiae

Comparison between the disintegration of S cerevisiae as measured by total count, extinction and turbidity By use of Fig 20 (Edebo 1961, page 13) logarithmic values are obtained corresponding to the extinctions and turbidities The decrease (—Δ log) of these logarithmic values by the disintegration process are then compared

Number of passages	Total count		Extinction 600 mμ			Turbidity		
	Log	log	Dilution 1 100	Log	log	Dilution 1 100	Log	log
0	9.66		1.40	11.74		91	11.95	
1	8.89	77	71	11.31	43	70	11.53	42
3	8.36	53	54	11.18	13	58	11.37	16
5			50	11.14	04	54	11.31	06
9			46	11.11	03	53	11.30	01

As was done with bacteria (Tables 4 and 5, *Edebo* 1961) the reduction of the extinction and turbidity has been compared with that of total count (Table 3) by using Fig 20 (page 13, this issue) The logarithmic decrease (—Δ log), calculated from extinction and turbidity values, is less than the decrease of the logarithms of the total count The explanation is that, when the cells are disintegrated, the cell walls still absorb and scatter light although to a reduced extent The reduction of the turbidity measured after the fifth and the ninth pressings is very small and indicates that the disintegration and the aggregation tendency (Fig 6) then balance each other

When examined by the phase contrast microscope the disintegrated cells are found to be less refractile than intact cells It seems likely that the minute cell wall cracks visualized in Fig 9 do not allow a sufficiently rapid or complete escape of the cell contents to label the cell as empty

The photomicrographs show that as is the case with certain kinds of bacteria (*Edebo* 1961) the yeast cell wall is disintegrated into smaller units (Fig 12) and that a certain amount of clumping occurs after many pressings (Fig 6) However after nine pressings (Fig 11), many well shaped cell walls can still be recovered which means that the mechanical strength of the yeast cell wall is greater than that of Escherichia coli (*Edebo* 1961) The small particles most clearly seen in Fig 3 are probably the ones described as chondriosomes (mitochondria) by earlier investigators (*Northcote & Horne* 1952 *Ingram* 1955 *Lindegren* 1949 *Winge & Roberts* 1958) Their numbers decrease after many pressings which probably indicates that they are disintegrated by the pressings In this connection it is interesting to note that *Nossal* (1954) who prepared enzymatically active particles probably mitochondria from yeast cells by shaking them with glass beads for 10 30 seconds found a reduction of the particle size and a migration of enzyme activity from particles to supernatant after 90 seconds of shaking

The thin membranes seen in the electron photomicrographs may be single layered cell walls In some photographs (*e g* Fig 8) a double layered cell wall structure (*Northcote & Horne* 1952) may be seen in others (Figs 9 12) bud scars are recognized

After one pressing about 60 per cent of the nitrogen of the cells is in solution and it increases still more after repeated pressings According to *Northcote & Horne* (1952) the soluble part of the yeast cell does not exceed 50 per cent Our results do not contradict this because the particulate fraction is mostly polysaccharide and lipid in nature and its relative nitrogen content is consequently lower so that nitrogen determination would be expected to yield a poor correlation to dry weight measurements

The nitrogen content and the UV absorbing capacity of the supernatants and the viscosity of the whole suspensions are increased by many pressings which means that no significant precipitation of the released macromolecules occurs However since the relative increase of the extinction at 260 mμ is greater than the increase of soluble nitrogen and the extinction at 280 mμ the colloidal state of the macromolecules is probably affected to some extent Such an effect might also help to explain a certain clumping of the cells after many pressings

Aspergillus mycelium is rather easily disintegrated in the X press The hyphae are broken up into shorter units and the cell content often particulate is released into the surrounding medium (Fig 13) Similar results are obtained with Penicillium notatum mycelia

The disintegration of *Penicillium* conidia (Fig 14) does not seem to be affected by the addition of agents which reduce surface tension This suggests that the water in the very most intimate contact with the outside cell surface is not of particular importance for the disintegration

The disintegration of bacteriophages does not seem to be easily reproducible This is probably due to the fact that in order to avoid damaging the disc of the X press the pistons cannot be pressed fully to the bottom of the compartment As a consequence a small part of the material (less than 10 per cent) which is situated closest to the pistons is not pressed through the hole This material probably adheres to the working surface of the pistons throughout several pressings since the flow of the sample being pressed occurs over the surface of the disc on its way through the hole (*Edebo* 1960) When odd numbered pressings are performed the material is taken out from the pressing chamber which was empty at the beginning of the procedure All this material must therefore have been pressed at least once At even numbered pressings however some of the material may never have passed the hole of the disc This causes an inhomogenous distribution so that non disintegrated material may in some instances contaminate the sample

SUMMARY

A wide variety of cells are disintegrated when they are pressed in the X press at —25° C and 2000 atm through a hole a few millimeters wide This investigation demonstrates the versatility of the X press procedure Previous papers have demonstrated its use for disintegration of bacteria of many species pollen and animal tissue This communication concerns the disintegration of yeasts mycelia conidia and bacteriophages (T2)

Cell walls and subcellular particles of yeasts are most clearly visualized after one pressing By many pressings they are disintegrated into smaller units particularly so the particles When bacteriophages are freeze pressed the phage heads are disrupted and the tails are occasionally separated from the heads

REFERENCES

Adams M H Bacteriophages Interscience Publ Inc New York 1959

Edebo L A New Press for the Disruption of Micro Organisms and other Cells J Biochem Microbiol Techn Engg 2 453 1960

Edebo L. Disintegration by Freeze Pressing I Effects on Bacteria Acta path et microbiol scand nav 52 300 1961

Heden C G & Wyckoff R W G The Electron Microscopy of Heated Bacteria J Bact 58 153 1949

Ingram M An Introduction to the Biology of Yeasts Sir Isaac Pitman and Sons Ltd London 1955

Kellenberger E & Arber W Die Struktur des Schwanzes der Phagen T2 und T4 und der Mechanismus der irreversiblen Absorption Ztschr Naturforsch *10b* 698 1955

indegren C C The Yeast Cell Its Genetics and Cytology Educational Publ Inc, Saint Louis 1949
itchell P Biochemical Cytology of Microorganisms Ann Rev Microbiol *13* 407 1959
orthcote D H & Horne R W The Chemical Composition and Structure of the Yeast Cell Wall Biochem J *51* 232 1952
ossal P M Distribution of Enzymes in Cell Free Yeast Extracts Biochem J 57 62 1954
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DEPARTMENT OF BACTERIOLOGY, KAROLINSKA INSTITUTET, STOCKHOLM 60
SWEDEN (HEAD PROFESSOR BERNDT MALMGREN)

LYSIS OF BACTERIA

1 Influence of Enzyme Inhibitors on Sonic[1] Lysis

By

LARS EDEBO

Received 6 III 61

When suspensions of bacterial cells are subjected to sonic treatment they usually lyse In this communication lysis is defined as the optical clearing of the suspension (*Pethica* 1958) In fact the extent of disintegration is often determined by photometric and nephelometric measurements (*Harvey & Loomis* 1929, *Yen & Liu* 1934, *Liu & Yen* 1934, *Chambers & Weil* 1938, *Shropshire* 1947, *Hompesch* 1949, 1950, *Rotman* 1956, *Welsch* 1957, *Marr & Cota-Robles* 1957) It has also been found that the extent of the lysis is decreased by a number of chemical and physical agents, *e g*, heat, increased hydrogen ion concentration (*Hompesch* 1949, 1950, *Rotman* 1956), absence of electrolytes, and some chemical substances (*Hompesch* 1949, 1950, *Rotman* 1956 *Welsch* 1957) Since these agents are known to reduce enzyme activity in several cases, these findings have been considered as some indications that sonic lysis may be of autolytic nature Actually sonic treatment, which is known to cause streaming and alterations in the structure of Paramecium, Spirogyra, Elodea, erythrocytes (*Harvey* 1930 *Goldman & Lepeschkin* 1952, *Lepeschkin & Goldman* 1952, *Nyborg & Dyer* 1960), may destroy enzyme barriers of cells and make it possible for enzyme and substrate to react with each other so that breakdown occurs In order to test this hypothesis the influence of a number of enzyme inhibitors on the sonic lysis of Escherichia coli B was investigated

MATERIALS AND METHODS

a *Cultivation of Test Organisms*

In this investigation unless otherwise specified E coli B was grown as a 200 l batch culture in a salt medium containing sodium lactate and ammonium chloride as the sole carbon and nitrogen sources (*Friedlein* 1928

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1 The limit between sonic and ultrasonic waves is set at 16 000 cycles per second (*Grabar* 1953) since above this limit the waves are not perceived by the average human ear From a physical point of view this limit is arbitrary In many cases the results described here for sonic lysis are directly applicable to ultrasonic conditions

b *Disintegration*

A Raytheon model DF 101 10 kC, 250 watt sonic oscillator was operated at resonance frequency and maximum efficiency Ten ml of of the suspension to be treated were placed in the cup and treated for 5 minutes under continuous cooling (temp below + 8° C) When not being treated the suspensions were kept in ice water baths All the determinations were made as soon as possible and were com pleted within 6 hours However, the difference between the individual samples in a series very rarely exceeded 15 min

c *Lysis Estimation*

After sonic treatment the extinction at 600 mμ was measured in a Beckman B spectrophotometer

d *Enzyme Activity*

The reducing activity of bacterial suspensions treated with enzyme inhibitors but not subjected to sonic treatment was estimated as tetrazolium reducing capacity without adding any particular enzyme substrate (*Kopper* 1952 1954) Instead of 2 3 5 triphenyl tetrazolium chloride used by Kopper 2 (p iodophenyl) 3 (p nitrophenyl) tetrazolium chloride (INT) (Light and Co Ltd Colnbrook, England) was employed INT was preferred to triphenyltetrazolium chloride since the redox potential of the former which is in the region of —0 06 volts, is more suitable for bacteria mainly because it is more rapidly reduced (*Pearse* personal communication) A test tube with 1 ml of the bacterial suspension was incubated in a water bath at 37° C for 5 min 1 ml INT (100 μg/ml) was then added and the mixture left for another 15 min in the water bath Five ml of acetone were then added the tubes stoppered and vigorously shaken to extract all dye from the bacterial cells and centrifuged The extinction at 480 mμ of the clear supernatants were measured in the Beckman B spectrophotometer

water

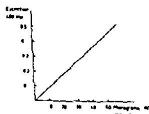


Fig 1
Relationship between amount of INT reduced and extinction at 480 mμ

e *Investigation Schedule*

Four ml samples of the enzyme inhibitors investigated (four times final con centration) were arranged in two fold serial dilutions Two ml of 0 8 M sodi acetate pH = 7 0

iodoacetamide Wh

glycylglycine buffe

(total count = 2

inhibiting effect o

and the whole mix

degree of INT redu

ibility of the inhibiting effect was tested one ml of 0 1 M cysteine pH = 6 7 wa

DEPARTMENT OF BACTERIOLOGY, KAROLINSKA INSTITUTET, STOCKHOLM 60 SWEDEN (HEAD: PROFESSOR BERNDT MALMGREN)

LYSIS OF BACTERIA

I. *Influence of Enzyme Inhibitors on Sonic*[1] *Lysis*

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Received 6 iii 61

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TABLE 1 a

Influence of Mercuric Acetate on Sonic Lysis and INT Reducing Activity without Addition of Cysteine

Final conc Hg Ac (M)	pH before sonic	pH after sonic	Extinction 600 mμ	INT reduced	
				Extinction 480 mμ	Micro-grams
10^{3}	6 9	6 8	1 55	0 004	< 1
5×10^{4}	7 1		0 565	0 005	< 1
$2\ 5 \times 10^{4}$	7 2		0 364	0 006	< 1
$1\ 25 \times 10^{4}$	7.2		0 304	0 019	2
$6\ 25 \times 10^{5}$	7.3		0 262	0 064	7
$3\ 13 \times 10^{5}$	7 3		0 213	0.267	30
1.56×10^{5}	7 3		0 203	0 305	34
0	7 3		0 200	0 316	36

TABLE 1 b

Influence of Mercuric Acetate on Sonic Lysis and INT Reducing Activity after Addition of Cysteine

Final conc Hg Ac (M)	pH before sonic	pH after sonic	Extinction 600 mμ	INT reduced	
				Extinction 480 mμ	Micro-grams
10^{3}	6 9	6 8	0 265	0 015	2
5×10^{4}	7 0		0 254	0 055	6
$2\ 5 \times 10^{4}$	7 1		0 248	0 233	26
$1\ 25 \times 10^{4}$	7 1		0 246	0 372	42
$6\ 25 \times 10^{5}$	7 1		0.220	0 339	38
$3\ 13 \times 10^{5}$	7 1		0 215	0 336	38
$1\ 56 \times 10^{5}$	7 1		0 207	0 450	51
0	7 1		0 180	0 400	45



Fig 3

Sonic lysis and amount of INT reduced in the presence of different concentrations of mercuric acetate without and with cysteine addition (——— sonic lysis without cysteine added x x — sonic lysis with cysteine - - - - amount of INT reduced without cysteine x - - x - - amount of INT reduced with cysteine)

added to each tube in the mercuric acetate series, while one ml of 0 5 per cent glutathione, pH = 7 5, was added to the p chloromercuribenzoate series After this last addition the suspensions were mixed and left for at least another 30 min in an ice water bath before sonic treatment and INT-reducing activity were estimated When the effects of iodine and potassium ferricyanide were investigated 4 ml samples of solutions of these substances were mixed with 3 ml 0 1 M sodium acetate pH = 7 0, and one ml of freshly cultivated bacteria (10^{11}/ml) The investigation schedule is visualized in Fig 2

In order to avoid lysis inhibition by hydrogen ions all experiments were performed close to neutrality

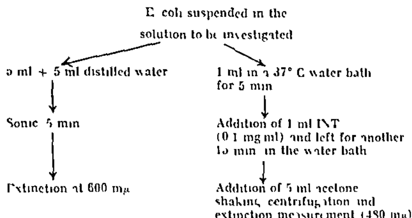


Fig 2
Investigation shedule for a comparison of the degree of sonic lysis and *INT-reducing activity*

RESULTS

The influence of mercuric chloride on sonic lysis and INT reduction was first tested since mercuric ions are known to combine with sulfhydryl groups which are essential for the activity of many enzymes (*Barron* 1951) Mercuric chloride inhibited sonic lysis, but the solutions of mercuric chloride initially were acid and difficult to buffer Therefore, mercuric acetate was used since it was easier to neutralize and somewhat more stable in neutral solutions The experiments listed in Table 1 and Fig 3 show that $6\ 25 \times 10^{-5}$ M mercuric acetate inhibited sonic lysis partially and that the inhibition was increased by higher concentrations of mercuric ions The decrease of INT-reduction became apparent at $3\ 13 \times 10^{-5}$ M mercuric acetate, and at $2\ 5 \times 10^{-4}$ M, the INT-reduction was completely inhibited, while the sonic lysis still was considerable

When 1 ml of 0 1 M cysteine was added to the suspensions, and the samples were left for another 30 min in an ice water bath before sonic treatment, almost all the sonic lysis was restored Only at the highest concentrations of mercuric acetate was the lysis little reduced However, at the two highest concentrations of mercuric acetate the INT-reducing *capacity* was still conspicuously inhibited after the addition of cysteine Addition of cysteine to a suspension where sonic lysis was inhibited by mercuric ions produced a rapid lysis

TABLE 3

Influence of Iodoacetamide on Sonic Lysis and INT-Reducing Activity

Final conc iodoacetamide (M)	pH before sonic	Extinction 600 mμ	INT reduced	
			Extinction 490 mμ	Micrograms
4 × 10^{-2}	7.3	0.267	0.013	2
2 × 10^{-2}	7.3	0.240	0.010	1
10^{-2}	7.3	0.230	0.019	2
5 × 10^{-3}	7.4	0.230	0.028	3
2.5 × 10^{-3}	7.4	0.230	0.024	3
1.25 × 10^{-3}	7.4	0.232	0.020	3
6.25 × 10^{-4}	7.4	0.232	0.027	3
0	7.4	0.231	0.485	54

TABLE 4

Influence of Iodine on Sonic Lysis and INT Reducing Activity

Final conc iodine (mg/ml)	pH before sonic	pH after sonic	Extinction 600 mμ	INT reduced	
				Extinction 490 mμ	Micrograms
0.5	6.8	6.8	0.323	0	0
0.05	6.7	7.0	0.074	0	0
0.005	6.6	6.8	0.063	0.025	3
0	7.0	6.9	0.061	0.343	39

TABLE 5

Influence of Potassium Ferricyanide on Sonic Lysis and INT Reducing Activity

Final conc $K_3Fe(CN)_6$ (M)	pH before sonic	pH after sonic	Extinction 600 mμ	INT reduced	
				Extinction 490 mμ	Micrograms
0.1	6.6	6.0	0.076	0.052	6
0.01	6.8	6.3	0.060	0.078	9
0.001	6.9	6.6	0.063	0.151	17
0.0001	7.1	6.9	0.060	0.270	30
0	7.0	6.9	0.061	0.343	39

The INT reducing capacity could also be inhibited by iodine (Table 4) (used as a solution of iodophor, "Jodopax", Ferrosan, Malmö, Sweden) and potassium ferricyanide (Table 5) within a wide range of concentrations without affecting the sonic lysis. Freshly grown bacteria were used in this experiment since the INT-reducing capacity of the frozen suspensions had decreased considerably.

A few other enzyme inhibitors were also tested (Table 6), *viz.*, 2,4-dinitrophenol, sodium azide, potassium cyanide and sodium arsenate. Sonic lysis occurred in each suspension even if the extent varied.

TABLE 2 a

Influence of p Chloromercuribenzoate on Sonic Lysis and INT Reducing Activity without Addition of Glutathione

Final conc p chlorom (M)	pH before sonic	pH after sonic	Extinction 600 mμ	INT reduced	
				Extinction 480 mμ	Micrograms
4 × 10 3	7 6	7 6	0 675	0 025	3
2 × 10 3	7 5		0 328	0 029	4
1 × 10 3	7 5		0 192	0 026	3
5 × 10 4	7 5		0 120	0 029	4
2 5 × 10 4	7 5		0 068	0 196	22
1 25 × 10 4	7 5		0 065	0 269	30
6 25 × 10 5	7 4		0 065	0 340	38
0	7 2		0 085	0 342	38

TABLE 2 b

Influence of p Chloromercuribenzoate on Sonic Lysis and INT Reducing Activity after Addition of Glutathione

Final conc p chlorom (M)	pH before sonic	pH after sonic	Extinction 600 mμ	INT reduced	
				Extinction 480 mμ	Micrograms
4 × 10 3	7 5	7 5	0 740	0 024	3
2 × 10 3	7 5		0 375	0 028	3
1 × 10 3	7 5		0 130	0 163	18
5 × 10 4	7 6		0 096	0 270	30
2 5 × 10 4	7 6		0 070	0 278	31
1 25 × 10 4	7 5		0 070	0 240	27
6 25 × 10 5	7 3		0 069	0 302	34
0	7 1		0 061	0 240	27

A more specific substance for the blocking of sulfhydryl groups, p-chloromercuribenzoate (Sigma Chemical Company, St Louis, Missouri), was also investigated for its inhibition capacity on sonic lysis and tetrazolium reduction The results are listed in Table 2 The INT-reducing capacity was decreased at 1 25 × 10^{-4} M and completely abolished at 5 × 10^{-4} M, but it could be partially reversed by the addition of glutathione The extinction at 600 mμ of the sonic treated suspensions was at a higher level at concentrations above 5 × 10^{-4} M This might, at least to a certain extent, be due to the fact that initially clear solutions of p chloromercuribenzoate in 0 01 M glycyl-glycine buffer (pH = 7 6) (*Smith & Smith* 1949) became turbid on standing

The results obtained with iodoacetamide, another reagent for blocking thiol groups, are summarized in Table 3 It appears that the INT-reducing capacity was completely abolished in the concentration range investigated without affecting the sonic lysis

260 and 280 mμ measured At concentrations about and above 10^{-3} M mercuric acetate turbidity appeared in the extracts and the organic UV-absorbing constituents were more easily sedimented

DISCUSSION

During the last three decades sonic and ultrasonic disintegration of cells has been successfully used as a tool for the preparation and isolation of bacterial components However, the disintegration mechanism is not yet quite clear One hypothesis (*Hompesch* 1949 1950, *Rotman* 1956 *Welsch* 1957) suggests that sonic lysis may be enzymatic in nature The sonic waves which are known to cause intracellular vibration in higher cells are said to destroy the cellular organization of enzymes activators inhibitors and substrates This should increase the possibilities for autolysis In order to test this hypothesis the influence of known enzyme inhibitors was tested

Several enzymes are known to be dependent on thiol groups for their action (*Barron* 1951) such that if the thiol group is blocked the enzyme activity is abolished Many substances are known which combine with thiol groups and their mechanisms of combination are also well known The mercuric ion was early known as a thiol group binding agent but it is less specific than others For a long time it has been used for antiseptic and disinfectant purposes The ion's bacteriostatic effect is thought to be due to combination with cellular thiol groups and is reversed by the addition of thiol substances (*Fildes* 1940) in a manner similar to the enzyme inhibition effect

The INT reducing activity was considered as a sign of diffusion of the enzyme inhibitor into the cell and its consequent inhibition of enzyme action Dehydrogenases which are the enzymes mainly responsible for tetrazolium reduction can scarcely be expected to be responsible for autolysis However INT reduction was selected because it was easy to determine and after all the nature of the enzyme(s) responsible for the sonic lysis was not known

The inhibition of INT reduction by mercuric acetate (Table 1 Fig 3) started at a concentration of 3.13×10^{-5} M and was complete at 2.5×10^{-4} M while the sonic lysis was slightly inhibited at 3.13×10^{-4} M but still was considerable at 5×10^{-4} M since the extinction of the suspension before sonic treatment was 0 60 at a 1 10 dilution and was reduced to 0 36a (undiluted) after sonic treatment When cysteine was added to the suspension containing mercuric acetate the INT reducing capacity was restored at the lower concentrations while it was still reduced at the higher concentrations An explanation of these results may be that at low concentrations mercuric ions attacked thiol groups essential for enzymatic activity without destroying the main structure of the protein At this concentration both the enzyme activity and the lysis inhibition were completely reversible upon binding of the mercuric ions

TABLE 6

Influence on Sonic Lysis of Enzyme Inhibitors not Blocking Thiol Groups

Chemical substance	Concentration (M)	pH before sonic	pH after sonic	Extinction 600 mμ
Dinitrophenol	0.1	6.4	6.6	0.940
Sodium azide	0.1	6.4	6.9	0.343
Sodium arsenate	0.1	7.9	7.9	1.180
Potassium cyanide	0.1	7.3	7.3	0.300
Potassium chlorate	0.5	6.8	6.3	0.505
Potassium chlorate	0.05	6.2	5.8	0.350
Distilled water		7.2	6.4	0.790

Since mercuric acetate was the only substance of the enzyme inhibitors investigated which inhibited both INT-reduction and sonic lysis, it was supposed that the precipitating action of mercuric ions might be responsible for the lysis inhibition effect. Bacteria examined by phase contrast microscopy after sonic treatment in mercuric acetate solutions showed, in addition to typical rods, also round, coccus-like particles which were thought to be fragmented cells. After addition of cysteine to bind the mercuric ions, the "cocci" disappeared and left only a few rods behind.

TABLE 7

Appearance of Turbidity and Reduction of Solubility after Addition of Mercuric Acetate to a Bacterial Extract

Final conc. $Hg\,Ac_2$ (M)	Turbidity	After centrifugation Extinction (1:50)	
		260 mμ	280 mμ
4×10^{-3}	64	0.038	0.030
2×10^{-3}	18	0.198	0.154
10^{-3}	6	0.335	0.235
5×10^{-4}	3	0.346	0.200
2.5×10^{-4}	3	0.342	0.193
1.25×10^{-4}	3	0.350	0.198
6.25×10^{-5}	3	0.354	0.203
0	3	0.332	0.188

The appearance of turbidity when mercuric acetate was added to a bacterial extract is demonstrated in Table 7. In order to prepare a cell extract *E. coli* B were disintegrated by freeze-pressing (*Edebo* 1960), suspended in 0.2 M sodium acetate, and centrifuged at 24,000 × g for 30 min. Four ml of the supernatant were mixed with 1 ml of a solution of mercuric acetate at twice the concentration desired. After standing in an ice-water bath for 30 min., the suspensions were centrifuged at 24,000 × g for 30 min. and the extinctions of the supernatants at

pension cleared Cysteine had probably bound the mercuric ions so that the precipitating action of the latter was taken away and the bacterial material dissolved

In order to make freeze pressed bacterial extracts turbid and precipitate, a higher concentration of mercuric ions was required than the minimal concentration which reduced the extent of sonic lysis This might be due to a low reaction velocity at limiting concentrations of mercuric acetate or to differences in the concentration of organic material between the bacterial suspension and the bacterial extract (*Edebo* 1961)

In a manner similar to the restoration of sonic lysis by cysteine, the material precipitated from the press extract by mercuric acetate was dissolved by the addition of that substance

Sonic lysis also occurred in the presence of the other enzyme inhibiting agents tested *viz*, dinitrophenol, potassium cyanide, sodium azide and sodium arsenate (Table 6), although the concentrations employed considerably exceeded those mostly used for enzyme inhibition studies (*Dixon & Webb* 1958) The absence of lysis inhibition by dinitrophenol, azide and arsenate, which interfere with aerobic phosphorylation processes indicates that this reaction a common energy-yielding process, is not involved in sonic lysis Azide and cyanide are powerful inhibitors of a great number of enzymes Sonic lysis also proceeded in the presence of high concentrations of these two The variations in the extent of sonic lysis (Table 6) were probably due to salt effects (*Edebo* 1961)

The high extent of lysis in the presence of potassium chlorate, a powerful oxidative agent and the increased rather than decreased extent of sonic lysis at pH 10 12 made the enzyme hypothesis still less probable because most enzymes are inhibited under these conditions

When a bacterial suspension sonic treated at lysis inhibiting conditions was adjusted to a lysis favouring state, the lysis occurred immediately at 0° C as well as at 30° C This was found for both the *pH dependent* lysis and when mercuric ions were bound by cysteine All of these facts do not support the enzymatic hypothesis of sonic lysis

Sonic treatment of a bacterial suspension in the presence of 0 1 M sodium citrate resulted in good lysis This suggests that a magnes[illegible]
ion [illegible]
inv [illegible]

da [illegible] shown as a disruption of the cell boundaries *i e* the cell wall and the cytoplasmic membrane (*Rotman* 1956) The nature of the second step after sonic treatment the [illegible]
has [illegible]

[illegible] many of the chemical agents employed are all known to decrease the solubility of organic macromole-

This might correspond to the number of ions which results in bacteriostasis At higher concentrations of mercuric ions thiol groups important for the tertiary structure of proteins might also be affected This could lead to damages which are not reversed after the removal of mercuric ions The decrease of the INT-reduction which remained is an expression for this The solubility state of the protoplasmic components, which is due to less subtle physical properties, should not be affected to this degree by slight structural changes

The inhibition of INT-reduction by p-chloromercuribenzoate showed great similarities to the inhibition by mercuric ions In this case the INT-reduction was restored by glutathione and by cysteine The extinction values of the sonic treated suspensions, however, give little information, because the clear solutions of p-chloromercuribenzoate became turbid to a degree corresponding to the sonic treated suspensions on standing However, at the highest concentrations of p-chloromercuribenzoate, where the INT-reducing capacity was completely abolished, the extinctions of the bacterial suspensions were reduced by sonic treatment to about one tenth of their original values The extinction after sonic treatment without enzyme inhibitor was higher in the series with mercuric acetate (Table 1) and iodoacetamide (Table 3) than with p-chloromercuribenzoate (Table 2), although the same bacteria were used This might be explained by the fact that sonic oscillation of the first two series was performed in 0 1 M sodium acetate, while 0 00125 M glycylglycine buffer was used in the series with p-chloromercuribenzoate Sonic lysis is suboptimal in 0 1 M sodium acetate (*Edebo* 1961)

The INT-reduction obtained after addition of cysteine and glutathione varied irregularly This may be due to a manifold effect by these substances

Iodoacetamide, iodine, and potassium ferricyanide inhibited the INT-reduction strongly in a wide concentration range without affecting the sonic lysis Only at high concentrations of iodine was the extinction at 600 mμ increased to a level which corresponded to the iodine present

The mode of action of the different thiol group binding reagents is very different Mercuric ions and p chloromercuribenzoate form reversible mercaptides, iodoacetamide forms irreversible alkylated compounds, and ferricyanide and iodine oxidize the thiol groups into –S S– bridges (*Barron* 1951) Mercuric ions together with other heavy metal ions, are also known to precipitate organic material, while none of the other compounds, except possibly p chloromercuribenzoate, would be expected to have that character It therefore seemed probable that a reduction of the solubility of the protoplasm was responsible for the inhibition of sonic lysis This hypothesis already proposed by *Rotman* (1956) is supported by phasecontrast microscopy There it was seen that sonic treatment, in the presence of mercuric ions disintegrated bacteria into smaller particles Upon addition of cysteine these particles were no longer visible in the phase contrast microscope and the sus-

Hompesch H Untersuchungen zum Mechanismus der bakteriziden Wirkung des Ultraschalls Nuovo cimento 7, Suppl 2 1950 p 498

Kopper P H Studies on bacterial reducing activity in relation to age of culture J Bact 63 639 1952

Kopper P H The assimilation of glucose by resting cells of E coli J Bact 67 507 1954

Lepeschkin W & Goldman D E Effects of ultrasound on cell structure J Cell Comp Physiol 40 383 1952

Liu S & Yen A C M Further studies on the effect of supersonic waves on bacteria Proc Soc Exp Biol and Med 32 485 1934

Marr A G & Cota Robles E H Sonic disruption of Azotobacter vinelandii J Bact 74 79 1957

[illegible]

Pearse A G F Intracellular localisation of dehydrogenase systems using mono tetrazolium salts and metal chelation of their formazans J Histochem and Cytochem 5 515 1957

Pethica B A Lysis by physical and chemical methods J gen Microbiol 18 473 1958

Ried W Formazane und Tetrazoliumsalze ihre Synthesen und ihre Bedeutung als Reduktionsindikatoren und Vitalfarbstoffe Angew Chem 64 391 1952

Rotman B On the mechanism of sonic lysis of bacteria J Bact 72 827 1956

Shropshire R F Turbidimetric evaluation of bacterial disruption by sonic energy J Bact 53 685 1947

Smith W F & Sm[illegible] as non

Welsc[illegible] sonique

Yen A C H & Liu S Effects of supersonic waves on bacteria Proc Soc Exp Biol and Med 31 1250 1934

cules Therefore, it seems reasonable to suppose, that, after disruption of the cell boundaries under conditions suitable for the dispersion of the protoplasmic gel, the gel will be transformed into a sol, the suspension will lyse, and the protoplasmic constituents will be soluble in a way excellently shown for Azotobacter vinelandii by *Marr & Cota-Robles* (1957) Enzymic reactions may possibly be involved in the dispersion of the protoplasm, but no data have hitherto been obtained which would support an enzymatic hypothesis in favour of a dispersion mechanism

SUMMARY

The influence of enzyme inhibitors on the sonic lysis of E coli B has been investigated Of the thiol binding reagents studied, only mercuric acetate inhibited sonic lysis On the other hand, iodoacetamide, iodine, and potassium ferricyanide had almost no effect on sonic lysis, despite the fact that the tetrazolium reducing capacity was completely removed at a comparatively wide range of concentrations In addition, when using mercuric acetate, the concentrations needed for inhibition of sonic lysis were greater than those required for enzyme inhibition

Other types of enzyme inhibitors such as azide, cyanide, dinitrophenol, and arsenate had no effect on sonic lysis at concentrations far exceeding those usually needed for enzyme inhibition

Since mercuric ions also precipitated bacterial protoplasm their inhibition of sonic lysis is postulated to be due to impaired conditions for dispersion of the cytoplasmic constituents after the bacterial cell wall has been disrupted by the sonic treatment This theory agrees well with earlier observations and may well be true also for other kinds of cell disintegration

REFERENCES

Barron E S G Thiol groups of biological importance Adv Enzymol *11* 201 1951

Chambers L A & Weil A J Immunological properties of a sonic extract of pneumococci Proc Soc Exper Biol and Med 38 924 1938

Dixon M & Webb E C Enzymes Longmans Green & Co London 1958

Edebo L A new press for the disruption of micro organisms and other cells J Biochem Microbiol Technol Engg 2 453 1960

Edebo L Lysis of bacteria II On the mechanism involved in mechanical lysis Acta path et microbiol scandinav 52 384 1961

Fildes P The mechanism of the antibacterial action of mercury Brit J Exp Path *21* 67 1940

Friedlein F Der quantitative Verwendungsstoffwechsel der Paratyphus B Bazillus des Bacterium coli und des Bacillus pyocyaneus Biochem Z *194* 273 1928

[illegible] Injury to living cells in standing sound waves

[illegible] d Phys *3* 191 1953

[illegible] al survey Biol Bull

Harvey E N & Loomis A L The destruct[illegible] acteria by high frequency sound waves J Bact *17* 373 1929

Hompesch H Über den Mechanismus der bakteriziden Wirkung der Ultraschallwellen Ultraschall in der Medizin S Hirzel Verlag Zurich 1949 p 218

and tetraethylpyrophosphate (*Rotman* 1956) are all known to diminish the extent of sonic lysis *Hompesch* (1950) also demonstrated that the efficacy of lysis inhibition by cations is related to their arrangement in the Hofmeister series: $Ca^{++} > Ba^{++} > Mg^{++} > Na^{+}$ However, no hypothesis applying to all collected data has been suggested and experimentally tested Since all the agents mentioned above may influence the solubility of the bacterial components, this study was designed to investigate the relationship between sonic lysis and the colloidal state of the bacterial protoplasm

MATERIALS AND METHODS

Cultivation

Escherichia coli strain B was chosen as the principal test organism It was cultivated on chemically def[illegible] in lar[illegible]

[illegible] ated in a 200 l culture in a medium [illegible] te and yeast extract as its main

Disintegration

Most disintegration exp[illegible] kc 250 watt sonic oscillator operate[illegible] [illegible]ency E. coli were sonic treated for 3 [illegible] treated for [illegible] °C. [illegible] ice [illegible] g in [illegible] (*Laeb* 1960 1961)

Measurements

The optical clearing (lysis) was estimated by measuring the extinctions at 600 mμ in a Beckman B spectrophotometer and the turbidities in a nephelometer (B Lange Berlin)

The pH (± 0 1) was measured with glass electrodes (pH meter 22 Radiometer Copenhagen)

After centrifugation in the cold at 25 000 × g for 30 min the extinction at 260 and 280 mμ was measured in a Beckman DU spectrophotometer

The state of aggregation of the bacterial [illegible]

[illegible] to stick to the tube walls Within 24 hours they had generally sedimented to the base of the tube [illegible] clear A partial [illegible] the cells while [illegible] Since the tenden[illegible]

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LYSIS OF BACTERIA

2 *Studies on the Mechanisms Involved in Mechanical Lysis*

By

LARS EDEBO

Received 21 iii 61

The information about the cellular structure of bacteria has accumulated rapidly during the last few years. Differentiation into cell wall, plasma (cytoplasmic) membrane, and protoplasm is generally accepted, and the importance of the cell wall as the structure responsible for the shape and for the mechanical protection of the cell is established. It is, for instance, known that the bacterial cell wall must withstand a considerable intracellular pressure (*Fischer* 1895, *Weibull* 1953, *Mitchell & Moyle* 1956). If it is disrupted, the protoplasm usually flows out of the cell and is dispersed in the suspension medium. This fact has been the basis for cell wall and protoplasm preparation techniques in the course of investigations on the chemical, enzymatic, antigenic, and genetic properties of microorganisms. Frequently sonic and ultrasonic treatment has been used to accomplish cell wall disruption. In the present investigation, sonic disintegration was generally preferred. The results obtained are, however, thought likely to yield information about the mechanisms common to other disintegration techniques.

When suspensions of bacteria sensitive to sonic oscillation are treated, they lyse, which is here defined as the optical clearing of the suspension (*Pelliica* 1958). It has been demonstrated (*Rotman* 1956, *Marr & Cota Robles* 1957) that the lysis is primarily due to a disruption of the cell wall, which, under proper conditions, is almost immediately followed by dispersion of the protoplasm. It has also been shown that sonic lysis is inhibited by several physical and chemical agents. Heat, increased hydrogen ion concentration (*Hompesch* 1950, *Rotman* 1956), removal of electrolytes by an ion exchange resin (*Rotman* 1956) and various substances such as formaldehyde (*Hompesch* 1950), dihydrostreptomycin sulfate (*Welsch* 1957), mercuric ions (*Edebo* 1961)

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up if the cells were percolated through a mixed bed ion exchanger before the pH adjustment. When these suspensions were sonic treated, the turbidity curve was shifted to a lower level parallel to the pretreatment curve.

The influence of the hydrogen ion concentration on the sonic lysis of Bacillus subtilis is illustrated in Fig. 2. At pH values below 6, the extent of sonic lysis was continuously decreased until it was quite inconspicuous at pH = 4.9. An extract (17 mg dry weight/ml), obtained after freeze-pressing, flocculated at pH 5.0 and the sonic-treated bacterial suspension showed slight sedimentation tendencies.

TABLE 1

Disintegration of E. coli B at pH 4 and pH 7.1 by different Disintegration Methods

Treatment	pH 4.0			pH 7.1		
	Extinction 600 mμ	Extinction supernat.		Extinction 600 mμ	Extinction supernat.	
		260 mμ	280 mμ		260 mμ	280 mμ
None	0.925	0.058	0.034	0.850	0.112	0.062
Sonic 3 min	0.720	0.072	0.044	0.067	1.180	0.678
Nossal 5 min	1.800	0.375	0.275	1.080	0.805	0.441
French press (×1)	0.950	0.062	0.032	0.185	1.600	0.637
X-press (×1)	*0.560*	*0.114*	*0.080*	*0.133*	*0.990*	*0.540*

The influence of pH on the extent of lysis obtained by various disintegration methods is shown in Table 1 for pH = 4 and pH = 7.1. In addition to sonic treatment, disintegration was done by high speed shaking with glass beads (*Nossal* 1953), by pressing in the liquid state (*French & Milner* 1955), and in the frozen state (*Edebo* 1960, 1961). It appears that, by the methods investigated, both the lysis and the UV-absorbing substances in solution after disintegration and centrifugation (Spinco rotor 40, 30,000 RPM, R av = 59,000 × g, 60 min.) were reduced at pH = 4 compared with those at neutral pH. The high extinction readings by the glass bead method were partly due to contamination with metal from the cups.

To test the reversibility of the inhibition of mechanical lysis by hydrogen ions, an E. coli suspension was adjusted to pH = 4 with 0.1 M citric acid and divided into three equal parts (25 ml each), which were then treated in the sonic oscillator (15 min., +8° C), French press (one pressing, room temperature), and the X-press (one pressing, 25° C), respectively. One ml of the treated suspensions was added to each tube of a series of citrate-phosphate buffers at various pH, and the pH and the extinction at 600 mμ of the suspensions were measured (Fig. 3). Lysis of all suspensions was observed at values above pH 5, i.e., in the same range of pH values required for the sonic lysis of bac-

RESULTS

Influence of Hydrogen Ion Concentration

The extent of sonic lysis at different hydrogen ion concentrations was investigated by adding, to a series of 10 ml portions of McIlvaine's citrate-phosphate buffers (*Gomori* 1955) at various pH, an equal volume (10 ml) of an E coli suspension twice washed in distilled water and then suspended in distilled water (subsequently called "aqueous E coli") The pH was determined before and after sonic treatment Of the 20 ml of suspension, seven were kept in an ice-water bath for the SR test Ten ml were sonic treated, and after the extinctions and the turbidities were measured, 7 ml were left for the SR test The results are shown in Fig 1 There the different parameters are plotted vs the pH and demonstrate that the extent of sonic lysis was decreased at pH values below 5 5 The lysis inhibition was successively increased at lower pH values until almost complete at pH = 4 5 (turbidity values before and after sonic treatment 91 and 86 respectively) When a bacterial freeze-pressed extract (18 mg dry weight/ml) was adjusted to various pH values by mixing 1 ml extract with 3 ml of 0 1 M sodium acetate buffer (*Gomori* 1955), turbidity and flocculation occurred at pH values close to the range where the extent of sonic lysis was decreased At pH values below 5 0 the floccules were very firm and separated from the rest of the material in a gel-like state

A large-scale culture (800 l) was grown for 15 hours and harvested by an Alfa-de Laval PX separator in which the cellular material was shot out of the spinning rotor After suspending those "mechanically strained" cells in solutions of different pH, they lysed spontaneously within an hour at pH values above 5, either at 0° C or room temperature The lysis was most rapid in the alkaline range It was also speeded

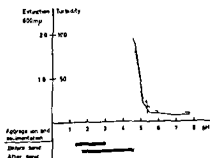


Fig 1

Influence of hydrogen ion concentration on the sonic lysis of E coli (extinction 600 mμ ——, turbidity – –) the sedimentation of the suspensions (complete sedimentation = solid bars partial sedimentation = lined bars) and the appearance of turbidity in E coli extracts 4 5 mg dry weight/ml (extinction 600 mμ - - - -)

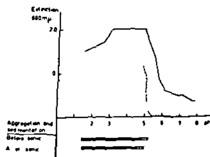


Fig 2

Influence of hydrogen ion concentration on the sonic lysis of B subtilis (extinction 600 mμ —) the sedimentation of the suspensions (complete sedimentation = solid bars partial sedimentation = lined bars) and the appearance of turbidity in B subtilis extracts 4 3 mg dry weight/ml (extinction 600 mμ)

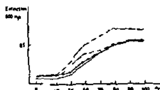


Fig 5
The degree of sonic lysis of E coli after heating in distilled water (——) and 0 1 M sodium chloride (- -), and after heating twice the concentration of bacteria in 0 2 M sodium chloride (-) and distilled water (-o-o-) and diluting with an equal volume of distilled water and 0 2 M sodium chloride respectively

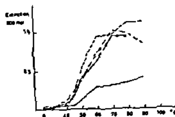


Fig 6
The appearance of turbidity after heating E coli extracts in distilled water (——), 1 M sodium chloride (- - -), 0 1 M sodium chloride (- - -), 0 1 M sodium acetate (-x x-) and 0 1 M sodium phosphate ( )

the concentration of bacteria were used and were suspended in both 0 2 M sodium chloride and distilled water, heated, and diluted in equal volumes of distilled water and 0 2 M sodium chloride respectively, before the sonic treatment Fig 5 demonstrates that the lysis inhibition by heat was most pronounced in suspensions heated in the presence of the salt

When smears of the sonic treated cells, both heated and non-heated, were fuchsin stained and examined, the degree of disappearance of distinctly stained cells was the same although the decrease in extinction was much less with the heated bacteria However, deeply stained granules were more abundant in smears from high temperatures

A suspension of freeze-pressed E coli cells in 1 M sodium chloride was centrifuged at 30,000 × g for 60 min The supernatant (after dialysis overnight against tap water, ca 15 mg organic substances ml), was adjusted to the various salt concentrations (pH 7 0 7 1) used in the sonic experiments on heated bacteria and exposed to the appropriate temperatures for 15 min The appearance of turbidity was measured as the extinction at 600 mμ after dilution 1 10 with the corresponding salt solution (Fig 6) Turbidity determinations yielded essentially the same results The curves in Fig 6 are not drawn beyond 90° C because the extracts were granular and sedimented rapidly above that temperature The development of turbidity after heating was least in the extracts with no added salts, where generally only a slight turbidity appeared even after heating at 100° C The turbidity was still less if the disintegrated bacteria were extracted with distilled water and no dialysis performed In contrast to these *findings*, in the presence of salts the turbidity was already significant at 50° C and subsequently rose even more

The influence of heat on the solubility and UV-absorption of bacterial extracts was also tested E coli disintegrated by freeze-pressing

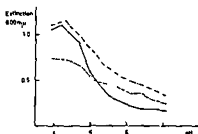


Fig 3
The lysis of E coli disintegrated at pH = 4 and subsequently adjusted to different pH-values Disintegration in the sonic oscillator (——), French press (– – –), and X-press (- - - -)



Fig 4
UV – absorbing material soluble at different pH-values Neutral extract prepared from B megaterium by sonic treatment (——) and freeze-pressing (- - - - -), and from E coli by sonic treatment -x—x) and freeze pressing (- x - - - x)

terial suspensions whose pH was adjusted to the final value before sonic treatment (Fig 1)

Extracts, in 0 03 M potassium phosphate, pH = 7, of Bacillus megaterium and E coli were prepared both by sonic treatment and freeze-pressing After centrifugation (30,000 × g, 30 min), the supernatants were adjusted to different pH values by ten drops of a mixture of 1 M H_3PO_4 and 1 M KH_2PO_4 The extinctions at 260 and 280 mμ of the supernatants were measured (Fig 4) after another centrifugation at 30,000 × g, 30 min The increase in sedimentation of the extracts at pH values around 5 was marked and agreed well with the lysis inhibition and the appearance of turbidity Fig 4 demonstrates the extinction at 280 mμ The extinction at 260 mμ showed similar behavior Only a slight increase in the 260/280 quotient was found as the hydrogen ion concentration increased

Heat

Heating of bacterial suspensions before sonic treatment considerably reduced the extent of sonic lysis (Fig 5) This was noted at temperatures as low as 45 to 50° C Solutions of various salts at a fourfold concentration *vs* the final were diluted from 25 ml to 100 ml with "aqueous E coli" suspensions (2 2 × 10^{10} bact /ml) Ten ml aliquots were pipetted into test tubes and held in an ice-water bath, then heated in a water bath at the desired temperature for 15 min , rapidly cooled in the ice-water bath, and held there until sonic treated It was observed that the extent of lysis inhibition caused by heat strongly depended on the nature of the suspension medium The lysis inhibition was always increased when salts were present (1 M and 0 1 M sodium chloride, 0 1 M sodium phosphate or 0 1 M sodium acetate) The results obtained with 0 1 M sodium chloride are shown in Fig 5 It was also interesting to see if the effect of electrolytes was caused only during the period of sonic oscillation or also during the period of heating To test this, twice

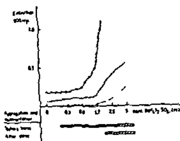


Fig 7
Ammonium sulfate

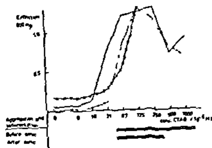


Fig 8
Cetyltrimetylammonium bromide
(CTAB)

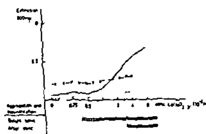


Fig 9
Lanthanum nitrate

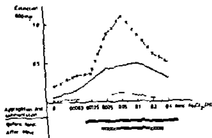


Fig 10
Manganous chloride

Figs 7 10
The effect of some salts on the extent of sonic lysis of E coli suspensions 0 5 mg bact /ml (———) sedimentation of the suspensions (complete sedimentation = solid bars partial sedimentation = lined bars) and the appearence of turbidity in extracts containing 0 9 mg (- -) and 9 mg (x - x x -) bacterial material/ml

and the sedimentation after sonic treatment were closely related to the appearance of turbidity in extracts, particularly so when similar concentrations of bacterial substances were used The ammonium sulfate and CTAB curves, representing the mixing with the concentrated press extract, rose continuously until 2 5 M and 0 01 M *respectively*, *after* which the curves reached outside the diagram At higher concentration, the extinction at 600 mμ could not be measured with the photometer employed

"*Salting-out*

Various ions and ion concentrations of the lyotropic series (*Hofmeister* 1888 1889 *Freundlich* 1903) influence the sonic lysis of bacterial suspensions differently This effect and the appearance of turbidity was also investigated (Figs 11 and 12) One ml "aqueous E coli" ($6 2 \times 10^{11}$ cells ml) was added to 9 ml of salt solutions with 10 9 the concentration listed, and the suspensions sonic treated The results, shown in Figs 11 and 12, were generally obtained at pH = 6 7-7 3 However,

were suspended in 0 1 M sodium acetate, pH = 7, homogenized, and centrifuged at 24,000 × g for 60 min The supernatant was then divided into equal samples and heated at different temperatures for 20 min, cooled, and the centrifugation repeated The supernatants were collected and their UV-absorbing capacity measured as listed in Table 2 The extinction at 280 mμ was continuously decreased with increased temperature which is thought to be mainly due to the removal of proteins The increase of extinction at 260 mμ by moderate temperatures can be explained by the single-stranding and hyperchromasi effect of heating on deoxyribonucleic acids (*Marmur & Lane* 1960, *Doty et al* 1960)

TABLE 2
Influence of Heat on Solubility and UV-Absorption of Bacterial Extracts

Temp (°C)	Extinction supernatant		
	260 mμ	280 mμ	Quotient 260 280
0	0 448	0 308	1 45
50	0 454	0 283	1 60
60	0 513	0 252	2 04
70	0 498	0 238	2 09
80	0 487	0 228	2 14
90	0 487	0 226	2 15

Selected "Specific" Salts

Some other salts were tested for their influence on the sonic lysis of E coli Ammonium sulfate, cetyltrimethyl ammonium bromide (CTAB), lanthanum nitrate and manganous chloride were selected because they cause protoplasmic precipitation

Ten ml of "aqueous E coli" (total count 1 5 × 10^{10}/ml, 0 9 mg dry weight/ml) were mixed with 5 ml of 0 02 M sodium phosphate buffer, pH = 7 when CTAB and $(NH_4)_2SO_4$ were tested and 0 2 M sodium acetate, pH = 7 for MnCl and $La(NO_3)_3$ plus 5 ml of a solution with four times the final concentration of the chemical to be investigated To exclude effects of the hydrogen ion concentration, the pH was checked before and after sonic treatment Except for the suspensions with the highest concentrations of ammonium sulfate, the pH was held between 6 7 and 7 2 none of the ammonium sulfate samples ever fell below 6 0 The SR was also observed The capacity of the above-mentioned salts to produce turbidity in initially clear bacterial extracts was tested both with an aqueous freeze pressed extract containing 18 mg dry weight ml and one with 1 8 mg ml Two ml of the salt solution, twice final concentration, were mixed with 2 ml extract The turbidity produced was measured as extinction at 600 mμ, just as the degree of sonic lysis was determined The results are illustrated in Figs 7 10 They demonstrate that in extracts prepared by freeze-pressing, the inhibition of sonic lysis

suspensions (pH = 6.1, final concentration 5 mg dry weight/ml) were adjusted to different concentrations of sodium chloride (1 M NaCl, pH = 7.3) and subjected to sonic oscillation. The viable counts after sonic treatment differed little and did not correspond to the extinction values. The lysis, as estimated by reduction in the extinction at 600 mμ, was strongly inhibited in the absence of salts. It was greatest at concentrations in the neighbourhood of 0.01 M sodium chloride. The table further shows that the addition of sodium chloride to the deionized bacterial suspension reduced the pH considerably. When it fell below 6.0, it was adjusted with 0.1 M sodium hydroxide before sonic treatment.

TABLE 3

Sonic Oscillation of Deionized Bacterial Suspensions to which various Concentrations of Sodium Chloride have been Added

NaCl conc (M)	Viable count (bact/ml)		pH of suspension				Extinction 600 mμ
	Before sonic ($\times 10^{-9}$)	After sonic ($\times 10^{-7}$)	Bact + NaCl	Drops of 0.1 N NaOH added	Before sonic	After sonic	
1	6.7	3.0	4.8	6	6.4	6.4	0.612
0.1	7.1	7.2	4.9	2+3	6.0	5.6–6.5	0.497
0.01	5.4	3.1	5.2	2	6.0	5.9	0.455
0.001	10	2.9	5.7	1	6.6	5.9	1.18
0.0001	11	4.2	6.1	0	6.1	5.7	1.46
0	14	5.4	6.3	0	6.3	5.7	1.40

DISCUSSION

The state of the bacterial constituents has been studied by determining

1. The degree of lysis obtained after sonic treatment of a bacterial suspension (measured as extinction at 600 mμ)
2. The sedimentation in the sonic treated suspension (SR)
3. The appearance of turbidity in initially clear bacterial freeze pressed extracts, generally measured as extinction at 600 mμ

As a rule the effects mentioned above were similarly influenced, i.e., when sonic lysis was inhibited, the sonic treated suspensions sedimented and the bacterial extracts became turbid.

The understanding of the stability of suspensions of bacterial cells has been facilitated by a colloid chemistry approach (cf. *Lamanna & Mallette* 1959). In order to easier understand the results obtained here, but at the risk of an oversimplification of a very complex problem, some rather general properties of biocolloids will similarly be considered in connection with the results obtained with bacterial constituents. Since the factors determining the stability of biocolloids are still not clearly

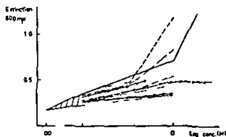


Fig 11
The effect of different anions on the extent of sonic lysis (cation = potassium) Citrate — — —, tartrate - -, acetate ——, sulfate — —·—, phosphate - - -, chlorate (0 1–0 5 M) and thiocyanate -o-o , chloride - - - -, iodide -x-x-, bromide - - -, nitrate — — —

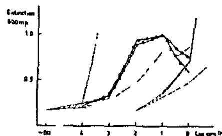


Fig 12
The effect of different cations on the extent of sonic lysis (anion = acetate) Lanthanum - - - - -, calcium -o o o barium x-x x- magnesium - - - - sodium — — — potassium ——— ammonium - - -

some non-buffered suspensions (bacteria in neutral solutions) were difficult to readjust to neutrality Therefore, the extinction of such suspensions was measured at the pH values reached, *viz*, pH = 6 5–6 6 in 1 M potassium bromide, chloride, iodide and nitrate, pH 6 2 in 0 1 and 1 M potassium acetate, pH 6 5 in 5 M potassium thiocyanate, and pH 6 1–6 5 in 0 001–0 1 M lanthanum acetate The lysis of E coli in sodium citrate-phosphate buffer was not conspicuously decreased at pH 6 0 (Fig 1), but the conditions are different at high salt concentrations The degree of sonic lysis was highest at relatively low salt concentrations Furthermore, the inhibition of sonic lysis was least pronounced in the suspensions of monovalent ions which have low salting-out effect (NH_4^+, K^+, Na^+, SCN^-, I^-, Br^-, ClO_3^-, NO_3^-, Cl^-) High concentrations of multivalent cations reduced the extent of lysis considerably, while the inhibition by multivalent anions was much less marked Sonic treatment of blank controls (pure salt solutions) never produced significant turbidity

The turbidity of bacterial extracts and the extent of their lysis by sonic treatment were inversely related At a salt concentration where the extent of sonic lysis was obviously diminished, the appearance of turbidity was enhanced Often, particularly when monovalent ions were investigated, no turbidity appeared after mixing the bacterial extract and the salt solution Turbidity could then be often produced by the addition of ethanol, and made it possible to exaggerate the differences between the different salts and salt concentrations

"Salting-in"

An "aqueous E coli" suspension which was repeatedly passed through an ion exchange resin column (Amberlite MB-3) exhibited very little lysis upon sonic treatment Table 3 demonstrates the killing effect and the degree of lysis produced when 10 ml of deionized bacterial

surface of the colloid If conditions exist where the solubility is impaired, the dispersion time would be expected to be further prolonged Differences in composition and concentration of the organic material might also have influenced the reactions

When conditions which inhibited the sonic lysis of bacteria were removed, e g, by neutralization or washing, the lysis was usually restored to a large extent The effects of heat and formaldehyde, denaturing and fixating agents, respectively, were, however, irreversible Heated suspensions which were sonic treated showed less tendency to sediment than those whose lysis was inhibited to the same extent by salts or pH The production of turbidity in bacterial extracts by heat showed a characteristic behavior Less turbidity appeared, when extracts in distilled water were heated, than when salts were present Heated bacterial extracts formed two distinct "phases", a clear supernatant and a "coagulated" bottom phase The ratio of the supernatant to coagulated part depended on the type of salt, its concentration, and the temperature The less striking appearance of turbidity in distilled water extracts may be explained by the well known fact that the presence of salts facilitates the heat coagulation of proteins (cf *Neurath et al* 1944) It may also be an expression of the protective action of nucleic acid for coagulation of protein by heat, which is most pronounced in the absence of salts (*Carter & Greenstein* 1945) The inhibition of sonic lysis by heat was greater when the bacteria were heated in 0 2 M sodium chloride than when heated in distilled water even though the suspensions were adjusted to the same salt concentration before sonic treatment This may be taken as an indication that the intracellular protoplasmic conditions were relatively easily influenced by the salt concentration in the suspending medium The electron photomicrographs of *Heden & Wyckoff* (1949) showed that bacteria heated in the presence of salts were more affected by heat than when heated in distilled water *Johnson & Gray* (1940) demonstrated that high concentrations of sodium chloride produced nuclear aggregation in Achromobacter fisheri, which could be reversed by removal of the salt This reaction was shown to be generally true for a wide range of microorganisms, whose metabolism was reduced (*Whitfield & Murray* 1956)

The "salting in" and the "salting out" effects on sonic lysis corresponded to those known to influence the solubility of macromolecules, i e at too high and too low ionic concentrations, both solubility and sonic lysis were impaired When washed E coli cells passed through the ion exchange resin column initially, the flow rate was less than on subsequent passage The pH of the suspension fell after the passages through the ion exchange resin from 6 9 to 6 1 If a small amount of sodium hydroxide was added to neutralize the suspension the flow time was increased When sodium chloride was added to the deionized bacterial suspensions the pH fell (Table 3) It is suggested that as the bacterial suspension passed through the ion exchange resin metal ions

established, the discussion will be confined to the relationships between the observations made within this investigation and results already presented by others concerning similar systems.

Most biocolloids are hydrophilic, *i.e.*, when dissolved in water, the molecules of the solvent are arranged at their surface. This arrangement is of great importance for the stability of the colloidal solution. Most cells and cellular constituents have their isoelectric points in the acid pH range. At the isoelectric point the stability of the colloids is decreased and the tendency of hydrophilic gels to imbibe water, swell, and eventually be dispersed is smallest (*Katchalsky & Eisenberg* 1950, *Jirgensons* 1958). When the bacterial suspension was disintegrated by sonic treatment at an increased hydrogen ion concentration the conditions for swelling and dispersion of the protoplasmic gel were consequently impaired. The stability of the already dispersed extract was similarly reduced. The effect was the reduction of sonic lysis, the aggregation and sedimentation in sonic treated suspensions, and the appearance of turbidity in the bacterial extracts. The solubility as measured as the UV-absorbing capacity after centrifugation was also reduced.

The bacterial substances are obviously inhomogeneous, *e.g.*, the isoelectric points of the proteins are generally at a pH-range higher than those of the nucleic acids. A change in the pH will therefore not only affect the state of the different substances separately but also their mutual relationship. The nature of the precipitation of nucleic acid with protamine is markedly influenced by the pH (*Miescher* 1897) and the precipitation of nucleic acid together with albumin is not obtained at neutral, but at low pH (*Hammarsten* 1924, *Hammarsten et al.* 1928). In this investigation only pH-determinations of the bulk of the liquid was done. The pH at the surface of the particles was not the same (*Pethica* 1958), so the numerical data presented cannot be representative of what exists at the macromolecular surface.

The effects of ammonium sulfate (Fig. 7), CTAB (Fig. 8), lanthanum nitrate (Fig. 9), and manganous chloride (Fig. 10) were similar to the *pH-effects. Ammonium sulfate is a common protein precipitant* and $La(NO_3)_3$ (*Hammarsten* 1924), $MnCl_2$ (*Korkes et al.* 1951) and CTAB (*Jones* 1953), are used as DNA precipitating agents. The agreement between sonic lysis, SR and appearance of turbidity was satisfactory particularly when suspensions with similar concentrations of bacterial substances were considered. The effects of CTAB showed a coinciding optimum at concentrations between 1.25 and 2.5×10^{-3} M and a similar optimum was between 0.05 and 0.1 M for $MnCl_2$. In the present experiments, the concentrations of these agents required to cause precipitation were generally higher than those which inhibited lysis. The observation (cf. *Jirgensons* 1958, *Lamanna & Mallette* 1959) that even under favourable conditions, the time for the dispersion of a gel is long may explain this. This period may be considered as the time required to form an ordered structure of the solvating water molecules on the

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(cf *Wilson & Miles* 1955) that the disinfecting effects of electrolytes may be due to their action on bacterial colloids

SUMMARY AND CONCLUSIONS

The effects of the hydrogen ion concentration, heating, "salting in" and 'salting out' effects, and several other agents on the sonic lysis of bacterial suspensions is closely related to the solubility of the bacterial protoplasm It is proposed that sonic lysis be regarded as a two step process *viz*

(1) structural damage to the cell boundaries, which, under favourable solubility conditions in the surrounding medium, permits the

(2) dispersion of the protoplasm

The first step is mainly dependent on the disintegration method employed and its efficiency while the second step is regulated by the surrounding medium

It is maintained that similar mechanisms will be critical for the lysis which is produced also by other mechanical disintegration methods The effects of the hydrogen ion concentration on bacterial lysis which follows high speed shaking with glass beads and pressing in the liquid and frozen state have been assessed As a result, it is obvious that the amount of bacterial material obtained after disintegration and extraction will be dependent on both the degree of disintegration produced and the solubility conditions for any cellular substances investigated

REFERENCES

v Angerer Uber die Arbeitsleistung eigenbeweglicher Bakterien Arch f Hygiene *83* 139 1919

Biernacki E Ueber die Beziehungen des Plasmas zu den rothen Blutkorperchen und über den Werth verschiedener Methoden der Blutkorperchenvolumbestimmung Zeitschr f physiol Chemie *19* 179 1894

Carter C F & Greenstein J P Protective effect of thymus nucleate on the heat coagulation of proteins J Natl Cancer Inst *6* 219 1945

Doty P Marmur J Eigner J & Schildkraut C Strand separation and specific recombination in deoxyribonucleic acids Physical chemical studies Proc Natl Acad Sci *46* 461 1960

Edebo L A new press for the disruption of micro organisms and other cells J Biochem Micr bi l Technol Engg 2 453 1960

Edebo L Disintegration by freeze pressing Acta path et microbiol scandinav 59 361 1961

Eisenberg P Untersuchungen über spezifische Desinfektionsvorgange II Mitteilung Ueber die Wirkung von Salzen und Ionen auf Bakterien Zbl Bakt Abt I *82* 69 1918

Fischer A Untersuchungen uber Bakterien Jahrb wiss Botanik 27 1 1895

French C S & Milner H W Disintegration of bacteria and small particles by high pressure extrusion In Colowick Kaplan Methods in Enzymology Vol I Academic Press New York 1955

Friedlein F [illegible]

Freun [illegible]

Fahraeus R The suspension stability of the blood P A Norstedt and Soner Stockholm 1921

(*e g* Na^+, K^+) were exchanged for hydrogen ions When neutral salts (*e g* , NaCl) were then added, the sodium ions displaced some of the hydrogen ions, and the hydrogen ion concentration of the solute increased It has frequently been observed that traces of ions are responsible for the stability of the colloidal particle (*Glasstone* 1948) The reduced extent of sonic lysis obtained after treatment of deionized E coli may thus be traced back to a lack of salts, which are necessary for the "salting in" effect

Earlier investigators maintain that the "salting out" effects on hydrophilic colloids by high concentrations of neutral salts is mainly due to a deterioration of the solvating layer Different anions and cations require different molar concentrations to cause precipitation of colloids The anions and cations have been arranged in series, the *Hofmeister* (1888, 1889) series, according to their precipitation efficacy The order of each series is not strict, but differs for each colloidal system and acidity of the medium The radius of the ions and their hydration seem to be responsible for these differences (cf *Glasstone* 1948, *Jirgensons* 1958) The inhibition of sonic lysis at high concentrations of neutral salts (Fig 11 and 12), correlated well with the arrangement in the Hofmeister series Some experiments have demonstrated that, after adjustment of the salt concentration in bacterial extracts, more turbidity developed in those solutions which most powerfully reduced the degree of sonic lysis

No clear distinction can be made between ammonium sulfate, lanthanum nitrate, manganous chloride, and those ions arranged in the Hofmeister series according to their effects on sonic lysis, sedimentation in sonic treated suspensions, and appearance of turbidity in freeze-pressed bacterial extracts At equimolar concentrations of the cations there was a valence effect which exceeded the effect which might have been expected from the higher ionic strength by the increased valency (Fig 12) The heightened effects of the multivalent anions at equimolar concentrations was, however, so small that it can be explained as due *to higher ionic strength These facts are consistent with the early re*cognised importance of the nature of the cations and the less significance of the anions for the precipitation of sols which migrate toward the anode (*Freundlich* 1903)

If the different ions were placed in series of decreasing ability to inhibit the sonic lysis of E coli, it would tentatively be the following for anions (Fig 11) Citrate > Tartrate > Acetate > Sulfate > Phosphate > Chlorate, Thiocyanate > Chloride > Iodide, Bromide, Nitrate, and for cations (Fig 12) (Lanthanum >) Calcium > Barium > Magnesium > Sodium, Potassium > Ammonium The arrangement of the cations shows some correlation with their bactericidal effects (*Holch Kiss* 1923) but the quantitative relationships are not so good The relationship of the anions to their bactericidal effect is still less satisfactory (*Eisenberg* 1919) It should be mentioned here that it has been proposed

(cf *Wilson & Miles* 1955) that the disinfecting effects of electrolytes may be due to their action on bacterial colloids

SUMMARY AND CONCLUSIONS

The effects of the hydrogen ion concentration, heating, "salting in" and "salting out" effects, and several other agents on the sonic lysis of bacterial suspensions is closely related to the solubility of the bacterial protoplasm It is proposed that sonic lysis be regarded as a two-step process *viz*

(1) structural damage to the cell boundaries, which, under favourable solubility conditions in the surrounding medium, permits the

(2) dispersion of the protoplasm

The first step is mainly dependent on the disintegration method employed and its efficiency while the second step is regulated by the surrounding medium

It is maintained that similar mechanisms will be critical for the lysis which is produced also by other mechanical disintegration methods The effects of the hydrogen ion concentration on bacterial lysis which follows high speed shaking with glass beads and pressing in the liquid and frozen state have been assessed As a result, it is obvious that the amount of bacterial material obtained after disintegration and extraction will be dependent on both the degree of disintegration produced and the solubility conditions for any cellular substances investigated

REFERENCES

v Angerer Uber die Arbeitsleistung eigenbeweglicher Bakterien Arch f Hygiene *88* 139 1919

Bernaef ...

ül

Z

Carter

c ... Cancer Inst 6 219 1945

Doty P Marmur J Eigner J & Schildkraut C Strand separation and specific recombination in deoxyribonucleic acids Physical chemical studies Proc Natl Acad Sci *46* 461 1960

Edebo L A new press for the disruption of micro organisms and other cells J Biochem Microbiol Technol Engg 2 453 1960

Edebo L Disintegration by freeze pressing Acta path et microbiol scandinav *52* 361 1961

Eisenberg P Untersuchungen über spezifische Desinfektionsvorgange II Mitteilung Ueber die Wirkung von Salzen und Ionen auf Bakterien Zbl Bakt Abt I *82* 69 1918

Fischer A Untersuchungen uber Bakterien Jahrb wiss Botanik 27 1 1895

French C S & Milner H W Disintegration of bacteria and small particles by high pressure extrusion In Colowick Kaplan Methods in Enzymology Vol I Academic Press New York 1955

Friedlein F Der quantitative ...

des Bacerium coli und de...

Freundlich H Uber das Ausfal...

physik Chem 44 129 190

Fåhraeus R The suspension stability of the blood P A Norstedt and Soner Stockholm 1921

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BRIEF REPORTS

PRELIMINARY REPORT ON THE FINDINGS OF PRECIPITATING ANTIBODIES AGAINST AN EXTRACT OF HUMAN BRAIN TISSUE IN INFANTILE SPASMS WITH HYPSARHYTHMIA

By Trond Reinskou

Infantile spasms with hypsarhythmia is a peculiar form of epilepsy in infancy and early childhood which is regarded as a clinico electroencephalographic entity. The disease is characterized by sudden lightning jerks which have been compared [illegible] as Salaam spasms. The electr[illegible]

[illegible]

of ACTH [illegible]

[illegible]

febrile illness in a previously healthy infant, and the successful treatment with ACTH [illegible]

[illegible]

In the present study sera from four patients with infantile spasms were tested for precipitating antibodies against saline extracts of human brain tissue by Ouchterlony's double diffusion method in agar gel (*Ouchterlony* 1958). The patients, aged 6 to 13 months, had all been admitted to Pediatric Department, Rikshospitalet, Oslo, Norway, where the diagnosis was made. Control sera were obtained from normal infants and children of the same age group, from children with other types of epilepsy, from normal adults and from adults with other diseases usually classified among the autoimmune disorders. The source of antigen was saline extracts of human brains obtained at autopsy from patients without recognized brain disease. The brains were obtained within 24 hrs. after death. The meninges were peeled off, and the brain and cerebellum were homogenized in a Waring blendor after addition of an equal volume of physiological saline solution. The homogenates were centrifuged at 25 000 r.p.m. (41 190 g) for 60 minutes, and the supernatants were stored at −20° C until used.

TABLE 1. *Tests for Precipitation Reactions in Agar between Sera and Saline Extracts of Human Brain*

Origin of sera	Number	Reactions	
		Positive	Negative
Infantile spasms	4	4	0
Controls			
Normal children	4	0	4
Epileptic children	9	0	9
Normal adults	5	0	5
"Autoimmune" diseases (LED, chronic thyreoiditis, rheumatoid arthritis, chronic hepatitis)	10	0	10

The reactants were filled into circular wells in an agar gel containing 1.5 per cent agar in 0.85 sodium chloride with 0.02 per cent sodium merthiolate. The plates were kept at 37° C in a humid chamber. The reactions were fairly weak and appeared

[illegible]

Glasstone S Textbook of physical chemistry D van Nostrand Co New York 1948
Gomori G Preparation of buffers for use in enzyme studies In Colowick Kaplan Methods in enzymology, Academic Press New York 1955
Hammarsten E Zur Kenntnis der biologischen Bedeutung der Nucleinsaureverbindungen *Biochem Zeitschr 144* 383 1924
Hammarsten E & Hammarsten G Über Nucleinsaure Eiweissverbindungen Acta Med Scand *68* 199 1928
Hofmeister F Zur Lehre von der Wirkung der Salze Arch exp Path Pharmak *24* 247 1888 *25* 1 1889
Homjesch H Untersuchungen zum Mechanismus der bakteriziden Wirkung des Ultraschalls Nuovo Cimento Suppl 2 1950 p 498
Hool A E Beard D Taylor A R Sharp D G & Beard J W Isolation and characterization of T 2 bacteriophage of Escherichia coli J Biol Chem *165* 241 1946
Hotchkiss M Studies on salt action VI The stimulating and inhibitive effect of certain cations upon bacterial growth J Bact *8* 141 1923
Jirgensons B Organic colloids Elsevier Publishing Co 1958
Johnson F H & Gray D H Nuclei and large bodies of luminous bacteria in relation to salt concentration osmotic pressure temperature and urethane J Bact *58* 675 1949
Jones A S The isolation of bacterial nucleic acids using cetyltrimethylammonium bromide (Cetavlon) Biochim Biophys Acta *10* 607 1953
Katchalsky A & Eisenberg H Polyvinylphosphate contractile systems Nature *166* 267 1950
Korkes S del Campillo Alice Gunsalus I C & Ochoa S Enzymatic synthesis of citric acid IV Pyruvate as acetyl donor J Biol Chem *193* 721 1951
Lamanna C & Mallette M F Basic bacteriology Williams & Wilkins Co Baltimore 1959
Marmur J & Lane D Strand separation and specific recombination in deoxyribonucleic acids Biological studies Proc Natl Acad Sci *46* 453 1960
Marr A G & Cota Robles E H Sonic disruption of Azotobacter vinelandii J Bact *74* 79 1957
Miescher F Die histochemischen und physiologischen Arbeiten Vogel Leipzig *1897*
Mitchell P & Moyle Jennifer Osmotic function and structure in bacteria Bacterial Anatomy Sixth Symp Soc gen Microbiol 1956 p 150 Cambridge University Press
Nossal P M A mechanical cell disintegrator Austr J exp Biol *31* 583 1953
Pethica B A Bacterial lysis Lysis by physical and chemical methods *18* 473 1958
Rotman B On the mechanism of sonic lysis of bacteria J Bact 72 827 1956
Weibull C The isolation of protoplasts from Bacillus megaterium by controlled treatment with lysozyme J Bact *66* 688 1953
Welsch M Influence de la dihydrostreptomycine sur la desintegration sonique d'Escherichia coli C R Soc Biol (Paris) *151* 621 1957
Whitfield J F & Murray R G E The effect of the ionic environment on the chromatin structure of bacteria Canad J Microbiol *2* 245 1956
Wilson G S & Miles A A Topley and Wilson's Principles of Bacteriology and Immunity Vol I Edward Arnold London 1955

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after 2 7 days One serum gave two precipitation lines the other three gave only one When two positive sera were deposited in adjacent wells in the agar, the precipitation lines showed reactions of identity

Seven different brain extracts were tested Five of these gave negative reactions with all sera, the remaining two gave identical reaction patterns as listed in Table 1

All brain extracts contained small visible amounts of hemoglobin Controls with hemolysates, however, showed that the positive reactions were not due to the factor in normal sera which precipitates a component of lysed human red cells (*Peetoom et al* 1960)

The reactions described most probably have a specific relation to this particular disease and suggest that sera from patients with infantile spasms contain a precipitating antibody directed against a saline souble factor in human brain tissue Further attempts to identify this factor are in progress The reactions are most likely explained as expressions of a factor of major pathogenetic importance in this disease The possibility that they represent a secondary phenomenon still remains to be excluded

References 1 *Bosma J F* In Molecules and Mental Health p 186 ed F A Gibbs Lippincott Philadelphia 1959 —2 *Gibbs I A & Gibbs F L* Atlas of Electroencephalography vol 2 Epilepsy Addison Wesley Press Cambridge Mass 1952 —3 *Gastaut H et al* Pediatrie *14* 35 1958 —4 *Hansted C & Thygesen P* Nord Med [illegible] 5 1 1958 —6 *Peetoom F et* [illegible] *aucy Bauloye A* Acta neurol [illegible] m Meeting in Danish Pediatric [illegible] *rojaborg W & Plum P* Uge

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